REMARKS

Claims 1 - 27 are currently pending in the application. Claims 5, 6, 9, 10, 14, 15, 18, 19, 22 – 27 are canceled without prejudice. Claims 1 - 4, 7, 8, 11, 13, 16, 17, 20, and 21 are newly amended. Support is found throughout the specification and in the claims as originally filed. No new matter has been entered. No claims are added.

Applicants thank Examiner Anderson and Examiner Marschel for the Interview of 12 October 2006 in which the outstanding Office Action was discussed.

Formal Matters

The Office Action states that the IDS filed May 10, 2005 fails to comply with 37 C.F.R. §1.97(c) because it lacks the fee set forth in §1.17(p). Applicants wish to again respectfully point out that page 2 of the information disclosure statement filed May 10, 2005 contains authorization to charge the fee for filing the IDS to Deposit Account 16-0085, reference number 8654/2222, please see attached copy downloaded from PAIR.

CLAIM REJECTIONS

The Examiner notes in the instant Office Action that Applicant's arguments traversing the previous claim rejections under 35 USC 102(b) and 35 USC 112, first paragraph, set forth on pages 3 – 7 of the Office Action mailed on 29 July 2005 have been considered but are not found to be persuasive. The Examiner notes that the rejections are maintained for the reasons provided in the previous Office action mailed on 29 July 2005, with additional comment.

Rejection of Claims Under 35 U.S.C. §102(b)

The Office Action has rejected claims 1 – 27 under 35 U.S.C. §102(b) as being allegedly anticipated by Siemann et al. (abstract) and Siemann et al. (Int. J. Cancer; "the Siemann et al. paper"). Applicants respectfully traverse.

The 29 July 2005 Office Action asserts that the Siemann et al. abstract teaches "methods of treating sarcoma, breast, and ovarian tumors with a combination of DMXAA...and cisplatin or cyclophosphamide." The 29 July 2005 Office Action stated that the Siemann et al. abstract is being supplemented by the Siemann et al. paper, because the Siemann et al. paper allegedly

teaches what is meant by a "rodent tumor model" as stated in the Siemann et al. abstract. Applicants note the post filing date reference of the Siemann et al. paper. The instant Office Action argues that another reference may be used to explain (supplement) the teachings of the primary reference. The 29 July 2005 Office Action concluded that the Siemann et al. abstract thus teaches treating cancer by administering the claimed composition to a mammal.

Applicants submit that anticipation requires that the purported prior art reference disclose each and every limitation of the claim. *Atlas Powder Company et al. v. IRECO, Incorporated et al.*, 190 F.3d 1342, 1347 (Fed. Cir. 1999).

The Siemann et al. abstract does not as required by section 102 teach all the limitations of the instant claims. Specifically, there is no teaching or suggestion in the Siemann et al. abstract of a method or composition comprising DMXAA and gemcitabine as required by newly amended claims 1 - 4, 7, 8, 11, 13, 16, 17, 20, and 21, as well as originally filed dependent claim 12. Therefore, Applicants respectfully submit that the Siemann et al. abstract does not anticipate claims 1 - 4, 7-8, 11-13, 16-17 and 20-21.

Applicants acknowledge the ability of two or more references to be combined under certain circumstances to make a rejection under section 102. However, even the combined teachings of the post-filing date Siemann et al. paper and the Siemann et al abstract, do not teach a method for treating cancer in a mammal by administering the combination of DMXAA and gemcitabine *in vivo*, as required by the newly amended claims.

Solely for the purposes of expediting prosecution, claims 5, 6, 9, 10, 14, 15, 18, 19, and 22 – 27 have been canceled without prejudice by Applicants, making the rejection of these claims moot.

In view of these claim amendments and remarks, Applicants respectfully request that the instant rejection be reconsidered and withdrawn.

Rejection of Claims Under 35 U.S.C. §112, First Paragraph

The Office Action rejected claims 1 – 6 and 24 – 27 under 35 U.S.C. §112, first paragraph for alleged overbreadth. The Office Action asserts that while the specification is enabling for the treatment of a number of cancers, it does not "reasonably provide enablement for treating cancers in general." Applicants respectfully disagree and traverse the rejection.

Applicants have amended claims 1, 3, 7, 8, 11, 13, 16, 17, 20, and 21 to limit the claims to a method of treating a solid cancerous tumor with an effective amount of DMXAA or a pharmaceutically acceptable salt or ester thereof and "an effective amount of gemcitabine." Support for this amendment is found in the claims and throughout the specification, particularly in Example 2.

The amended claims are drawn to a method of treating a solid cancerous tumor using a combination of DMXAA and gemcitabine. One of skill in the art, given the disclosure of the instant specification and the level of knowledge and skill in the art, would be able to select a particular solid cancerous tumor of interest for treatment, use gemcitabine in combination with DMXAA, and determine whether the solid cancerous tumor is treated by the chosen combination.

The specification teaches solid cancerous tumors that can be treated according to the claimed invention. The specification teaches at page 22, line 25 to page 23, line 2, that the invention can be used for treatment of solid tumors that can include such cancers as non-small cell lung cancers, small cell lung cancers, breast cancer, cancer of the pancreas, ovarian cancer, colorectal cancer, prostate cancer, gastric cancer, testicular cancer, bladder cancer, colonic carcinoma, parvocellular and non-parvocellular bronchial carcinoma, carcinomas of the cephalic and cervical parts, carcinomas of the thoracic and abdominal regions, cervical and endometrial carcinomas, sarcomas, and melanomas. In particular, the Examples provide specific teachings of the ability of the methods of the invention to treat solid tumors using DMXAA in combination with gemcitabine to treat pancreatic carcinoma. Example 2, beginning on page 32, demonstrates that DMXAA in combination with gemcitabine demonstrates anti-tumor activity. The specification teaches one of skill in the art the *in vivo* tumor model to use to test the claimed combination, the method for preparation of DMXAA and gemcitabine stock solutions, and the mode and method of treatment for combination therapy. Example 2 sets out exactly how the solid cancerous tumor is grown in the mouse model, how the tumor is treated, and how the tumor is measured and how treatment is assessed (i.e. the advantage of the drug or the drug combination over untreated tumors). Additionally, Applicant points out that preparation of both DMXAA and platinum compounds, such as gemcitabine, are commonly known in the art, as referenced at page 22 of the specification (lines 14 - 21). On page 23, the specification teaches a

range of cancer cell lines for use in the animal models as taught in the Examples of the invention, to test the DMXAA and gemcitabine combination therapy.

The specification teaches that DMXAA has been shown to induce significant reduction in tumor blood flow (see page 1). It is well known in the art that solid tumors share the common feature of vascularization; a feature that may serve as a target for tumor treatment (See, e.g., Denekamp J. Endothelial cell proliferation as a novel approach to targeting tumour therapy. Br. J. Cancer, 45: 136-139, 1982.; Denekamp J. Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. Br. J. Radiol., 66: 181-196, 1993.).

The specification enables one of skill in the art to use gemcitabine in combination with DMXAA for treatment of a particular solid tumor. The art recognizes that gemcitabine is useful in the treatment of other solid tumor types, including pancreatic, gallbladder, head and neck and breast cancer (Rachamalla R. et al. Phase I dose-finding study of biweekly irinotecan in combination with fixed doses of 5-fluorouracil/leucovorin, gemcitabine and cisplatin (G-FLIP) in patients with advanced pancreatic cancer or other solid tumors. Anticancer Drugs. 2004 Mar;15(3):211-7). Further, the reference cited by the Office Action (Goodman & Gilmans's) teaches that conventional anti-cancer compounds (including those recited in the claims) can be used to predictably treat solid tumors including, but not limited to, neuroblastoma, breast tumor, ovarian tumor, lung tumor, Wilms' tumor, cervical tumors, testicular tumors, colon tumor, stomach tumors, pancreatic tumors, head and neck tumors, bladder tumors, small cell lung tumors, endometrial tumors, and thyroid tumors.

Thus, the specification teaches how to make and use the invention, and teaches further how one of skill in the art would test a combination of DMXAA and gemcitabine for its ability to treat any type of solid cancerous tumor. As acknowledged by the Office Action, the level of skill of one in the art is high, thus the level of experimentation required to carry out the full scope of the claimed invention is merely routine, and not undue.

The Office Action asserts that the specification is not enabling because the "prior art recognizes that no one compound or combination of compounds is capable of treating" all cancers. The claims relate to the treatment of a solid cancerous tumor by administering a combination of DMXAA and a known anti-cancer agent, gemcitabine. The claims do not require that gemcitabine in combination with DMXAA be able to treat all cancers. In addition,

Applicants respectively submit that the assertion in the Office Action that "the prior art recognizes activity of the claimed compounds against a limited number of cancer types" is misleading. As with this or any invention, it is the very fact that the prior art does not teach what is being claimed that makes the invention patentable. Moreover, the fact that a particular prior art reference reports on a particular combination of drugs recited in the claims relative to a particular cancer type (although, as noted above, Applicants do not concede that Siemann et al. teaches administration of the claimed combinations to a mammal to treat cancer) does not mean that the claimed compounds are "limited" to the treatment of just those types of cancers.

The Examiner argues that the Applicant is not required to enable each and every single embodiment encompassed by the claims, but must enable a sufficient number to be reasonably representative of that which is claimed. The Examiner argues that it would require undue experimentation for one of ordinary skill in the art to determine how the present invention could be used to treat all forms of solid cancerous tumors. It is unclear to the Applicant, given the disclosure of the specification that clearly enables the method for treating a solid tumor with the DMXAA and gemcitabine combination, what is a sufficient number of tumor types would be to enable one of skill in the art to perform the invention without undue experimentation. As such, the MPEP states that the determination that "undue experimentation" would have been needed to make and use the claimed invention is not a single, simple factual determination. Rather, it is a conclusion reached by weighing all the above noted factual considerations. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

Taken together, the teachings of the specification and knowledge of those of ordinary skill in the art enable one of skill in the art to practice the full scope of the claimed invention without having to resort to undue experimentation. The rejection of Claims 5-6 and 24-27 is moot, these claims having been newly cancelled by Applicants. Applicants accordingly request that the rejection of the remaining instant claims be reconsidered and withdrawn.

Conclusion

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with

Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

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Short Communication

ENDOTHELIAL CELL PROLIFERATION AS A NOVEL APPROACH TO TARGETING TUMOUR THERAPY

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THE proliferation rate of vascular endothelial cells in tumours exceeds that in most adult normal tissues by a very large factor, often greater than 20 (Figure). This enormous differential offers a potential route for aiming tumour therapy at solid tumours by means of a targeted systemic toxin, with little risk of damage to most of the normal tissues.

It has long been recognized that one of the major differences between solid tumours and adult normal tissues is the pattern and rate of development of the vascular network. New vessel formation in tumours is rapid, but it is insufficient, particularly in a 3-dimensional arrangement, to provide an adequate nutrient supply to all the tumour cells (Hirst *et al.*, 1982). For this reason many tumour cells are non-proliferating because of nutrient deprivation, and hypoxic because of oxygen depletion by the metabolizing tumour cells around each capillary. This produces resistance to both chemotherapy and radiotherapy. A great deal of research is devoted to finding ways of improving the oxygenation, proliferative status and drug delivery in such tumours.

Folkman and co-workers (Folkman *et al.*, 1971; Folkman, 1974, 1975) have recognized the special ability of tumour cells to promote new vessel formation via the tumour angiogenesis factor (TAF). They have proposed methods of preventing tumour growth by interfering with angiogenesis, including an immunological technique for inactivating TAF by producing an anti-TAF antibody which would prevent further capillary proliferation.

Elsewhere, little attention has been paid to the enormous *differential* between the proliferation characteristics of the tumour vasculature and the normal tissue vasculature. Tannock (1970), Hirst & Denekamp (1979) and Hirst *et al.* (1982) have compared the proliferation rate of the capillary endothelium in tumours with that of the tumour cells themselves. They concluded that the rate of endothelial proliferation limits tumour cell production even though as many as 18% of the endothelial cells can be in DNA synthesis at any one time.

Other studies have concentrated on the very low proliferation rate of endothelial cells in a variety of normal tissues (see reviews by Tannock & Hayashi, 1972; Hirst *et al.*, 1980). However, the remarkable difference in proliferation rates between endothelium in tumours and in normal tissues does not seem to have been previously commented upon or identified as a potential route for *directing* therapy at a tumour.

The Table summarizes the published labelling index (LI) for normal tissue and tumour endothelium, and illustrates the large difference between them. The values shown were obtained by scoring autoradiographs of tissues sampled $\frac{1}{2}$ h–1 h after a single injection of the DNA precursor, [^{3}H]dT. A mean LI of 0·6% is obtained for normal tissue endothelium, by averaging all the values reviewed by

TABLE.—*Proliferation of vascular endothelium in tumours and normal tissues*

Normal tissues	LI (%)	Potential turnover time (Days)	Volume doubling time (Days)	Reference
Aorta (Mean of 7 studies)	0·61	55	∞	Reviewed by Tannock & Hayashi, 1972; Hirst *et al.*, 1980
Arteries & Veins (Mean of 6 studies)	0·82	41	∞	
Capillaries (Mean of 14 studies)	0·56	60	∞	
Tumours		(Hours)		
C3H mammary carcinoma	11·4	55	2·5	Tannock, 1970
C3H carcinoma KHH	17·7	45	5	Hirst *et al.*, 1982
C3H carcinoma KHU	17·9	45	6	
WHT carcinoma RH	4·5	178	13	

The potential turnover time is calculated as $\lambda Ts/LI$ (assuming $\lambda = 0{\cdot}8$ and $Ts = 10$ h) where λ is a correction factor for the non-linear distribution of cells throughout the phases of the cell cycle (Steel, 1968).

Tannock & Hayashi (1972) and Hirst *et al.* (1980) for a wide range of normal tissues. It does not differ much between major vessels and capillaries. Most authors have indicated extremely low proliferative activity in normal tissue endothelium, with half the studies showing an LI < 0.25%. The highest recorded value is 3·4% for adult rat mesenteric vessels (Crane & Dutta, 1964) but this contrasts markedly with 0·45% for the same tissue in mice (Hirst *et al.*, 1980).

Fewer data have been published for tumours; these are also summarized in the Table. The lowest LI is 4·5% for the exceptionally slow-growing mouse tumour CA RH (Hirst *et al.*, 1982) and two of the tumours have values approaching 18%. This difference between tumours and normal tissues is illustrated even more dramatically in the Figure. Some additional data are included for rat and mouse tumours (Denekamp & Hobson, in preparation) which show a range from 3·5% for lymphoma KHAA (which grows by infiltration rather than by evoking a neovasculature) to 32·5% for the rapidly growing rat fibrosarcoma RIB_5. The mean of the endothelial LI values in tumours is more than 20-fold that for normal tissues and there is hardly any overlap of the histograms.

With the advent of sophisticated immunological techniques, including monoclonal antibodies, it seems likely that

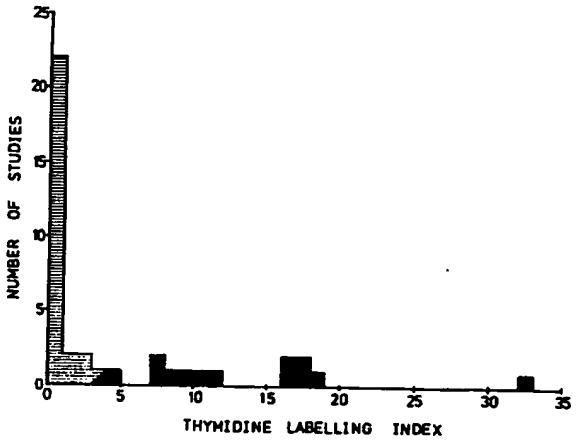

FIGURE.—Vascular endothelial cell proliferation. A histogram showing the number of studies in normal tissues and in tumours with particular values for the labelling index of endothelial cells ½–1 h after a single injection of [^{3}H]dT. Half of the normal-tissue studies ▤ show values below 0·25% with a mean LI of 0·6%. The range for tumours ■ is from 3·5–32·5% with a mean of 13·5%. There is a very large differential in these LI values with almost no overlap.

this tremendous proliferative differential could be exploited. An immuno-histochemical technique is already in use to identify endothelial cells in histological preparations, by means of Factor VIII antigen (Hoyer *et al.*, 1973) and monoclonal antibodies have recently been raised against this antigen (Sola *et al.*, 1981). It should be possible to conjugate an S-phase-specific chemotherapeutic agent with an endothelial-cell-specific antibody, which could result in a large

degree of cell killing in tumour capillaries with very little damage to any normal tissue vessels. It is also conceivable that antibodies could be raised more specifically against proliferating endothelial cells, as distinct from all other endothelial cells. These could then be conjugated with any potent cell toxin (*e.g.* abrin or ricin) which would be released at the desired site of action. Proliferating endothelial cells can be produced very readily *in vivo* as a granulomatous response to an irritant; this would provide a richer and easier source of dividing endothelium than tumour vessels for raising antibodies This approach, directing tumour therapy via the proliferating endothelium, would avoid the necessity for identifying a tumour-specific antigen. It could provide a means of attacking solid tumours, including small metastases, via a universal pathway which is already known to be the weak point in tumour development. It would need to be used in combination with radiation and/or chemotherapy, and the sequencing would be very important. As vessels become occluded or collapse the surrounding cells would become more deficient in nutrients and oxygen and hence more chemo- and radiation-resistant. The possible influx of endothelial cells from the general circulation would also need to be studied, but this seems to be a relatively slow process (Hobson *et al.*, 1980).

Potential side effects, of course, would need a very thorough investigation in animals. Fresh wound tissue, premenopausal endometrium and placenta, are all tissues in which a highly proliferative endothelium will exist, and these would probably be at risk. Patients undergoing surgery, pregnant women, or those in the initial proliferative phase of each oestrus cycle, would therefore be unsuitable for this form of therapy. However, anti-proliferating-endothelium therapy (APET) would be likely to be of only a few days duration to injure most of the tumour endothelium, as judged from the potential turnover-time estimates for tumour endothelium, at least for rodent tumours (Table). The demonstration of equally high LIs for endothelium in human tumours would of course be crucial for this approach to have a clinical future. This information should be readily obtainable with biopsy material and *in vitro* incubation (Denekamp & Kallman, 1973).

A small loss of endothelial cells in normal tissues is unlikely to be of major significance. Radiation studies on mesenteric arterioles have shown that the endothelium can be gradually depleted to 10–20% of its normal cell number without any thrombosis or failure of vessel function. (Hirst *et al.*, 1980). This functional reserve probably results from the well-developed sub-endothelial layers in normal blood vessels, which are notably lacking in most tumour vessels. It is not known how catastrophic the loss of 50% or 90% of the *tumour* endothelial cells would be, but it is possible that the already poor nturient supply would collapse and lead to massive tumour-cell necrosis.

We are currently studying a variety of other tumours and normal tissues. We are using repeated doses of [^{3}H]dT to determine how many sequential injections are needed to label all the endothelial cells. Preliminary data indicate that the 20-fold difference between tumour and normal vessels persists after one week of continuous labelling (Denekamp & Hobson unpublished). From these data it should be possible to predict how long an APET treatment with an S-phase-specific endothelial toxin would be needed to affect a given proportion of the endothelial cells in tumour capillaries.

I am very grateful to Mrs B. Hobson for her excellent assistance and to the Cancer Research Campaign for their financial support.

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Phase I dose-finding study of biweekly irinotecan in combination with fixed doses of 5-fluorouracil/leucovorin, gemcitabine and cisplatin (G-FLIP) in patients with advanced pancreatic cancer or other solid tumors

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This phase I trial was initiated based on encouraging clinical data with 5-fluorouracil (5-FU)/leucovorin (LV), gemcitabine and cisplatin (G-FLIP) in the therapy of solid tumors. In this trial, G-FLIP has been modified to facilitate outpatient administration and to optimize sequence-dependent synergistic activity. Treatment consisted of biweekly (once every 14 days) cycles of sequential gemcitabine 500 mg/m^2, irinotecan per dose escalation schedule, bolus 5-FU 400 mg/m^2 and LV 300 mg on day 1 followed by a 24-h 5-FU infusion 1500 mg/m^2, followed by cisplatin 35 mg/m^2 on day 2. The irinotecan starting dose was 80 mg/m^2 and escalated by 20 mg/m^2 in cohorts of three patients until the maximum tolerated dose (MTD) was defined. Twenty-three patients were enrolled (13 men/10 women) with the following cancers: 11 pancreatic, five gallbladder, three squamous cell carcinoma of the head and neck, one hepatocellular carcinoma, one melanoma, one gastric, and one breast cancer. Median patient age was 63 years (range 44–78) and median Karnofsky performance status (KPS) was 80. Patients received a median of 8 cycles (range 1–16) over five irinotecan dose levels (80, 100, 120, 140 and 160 mg/m^2). Dose-limiting toxicity consisting of grade 3 nausea/vomiting despite aggressive anti-emetic therapy occurred in one patient at dose level 1 and three patients at dose level 3. Grade 3–4 hematological toxicities per patient consisted of thrombocytopenia (3%), anemia (6%), thrombosis (23%), neutropenia (16%) and neutropenic fever (10%). Of 18 patients evaluable for response, one complete response (pancreatic) and eight partial responses (three gallbladder, two pancreatic, two head and neck, and one breast) were attained. Seven patients had disease stabilization (five pancreatic, one hepatocellular and one gastric) for a median of 16 weeks (range 10–22). Median time to disease progression among all 23 patients enrolled to the phase I portion of the trial was 20.5 weeks (range 4–37). We conclude that G-FLIP is a novel outpatient chemotherapy regimen with acceptable toxicity at the maximum tolerated irinotecan dose of 120 mg/m^2. The phase II trial of G-FLIP using an irinotecan dose of 120 mg/m^2 for patients with metastatic pancreatic cancer is ongoing. *Anti-Cancer Drugs* 15:211–217 © 2004 Lippincott Williams & Wilkins.

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Keywords: 5-fluorouracil, cisplatin, gemcitabine, irinotecan, leucovorin, pancreatic cancer, phase I trial

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Introduction

The novel combination of gemcitabine, 5-fluorouracil (5-FU) bolus plus infusion, irinotecan and cisplatin (G-FLIP) was developed to approximate known sequence-dependent activity while minimizing sequence-dependent toxicity among the four drugs. A retrospective analysis of a similar regimen containing these four drugs demonstrated encouraging activity and survival outcomes in heavily pretreated patients, all with metastatic pancreatic cancer, thereby encouraging phase I and II development of the regimen with a simplified 5-FU schedule [1].

Irinotecan (CPT-11, Camptosar) is a water-soluble topoisomerase I inhibitor with a unique mechanism of action of reversible inhibition of DNA topoisomerase I and a broad range of antitumor activity as a single agent [2]. Topoisomerase I inhibitors also may interfere with processes involved in DNA repair and enhance cytotoxicity when combined with DNA-damaging agents such as cisplatin. Overlapping single-agent activity, non-overlapping toxicities, and a lack of cross-resistance and potential synergism in preclinical studies provide the rationale for combining irinotecan and cisplatin [3–5]. Several phase I trials have demonstrated the feasibility of combining these two drugs in a variety of dosages, schedules and sequences. Phase I and II data support total monthly cisplatin doses of 60–80 mg/m^2, and monthly irinotecan doses between 180 and 280 mg/m^2 independent of

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dose scheduling (i.e. weekly, biweekly, monthly dosing) [6]. There is clinical evidence suggesting reversal of cisplatin resistance when it is combined with irinotecan [7].

Gemcitabine is a cell-cycle-specific pyrimidine nucleoside analog that undergoes intracellular phosphorylation to form active di- and triphosphates. This process is dose rate dependent so the effectiveness of gemcitabine may be improved by altering the standard infusion schedule to a fixed-dose rate [8]. Gemcitabine cytotoxicity correlates with its incorporation into genomic DNA thereby inhibiting DNA synthesis.

5-FU is an anti-metabolite that also requires intracellular activation to inhibit DNA synthesis. Preclinical and clinical data have reported cytotoxic synergy between gemcitabine and 5-FU in pancreatic and ovarian cancers [9]. Several phase I–II studies using different infusion schedules of 5-FU in combination with gemcitabine have reported potentially additive activity for the combination in pancreatic and renal cell carcinoma with tolerable toxicity profile [10–13].

Irinotecan can be combined safely with gemcitabine and the doublet has demonstrated response rates of 20–30% in patients with locally advanced or metastatic pancreatic cancer. Toxicities include grade 3–4 neutropenia and grade 3 diarrhea [14,15]. Combinations of cisplatin with either 5-FU or gemcitabine have been studied extensively in various malignancies including esophageal, lung, cervix, head and neck, and urothelium.

The G-FLIP regimen is based on laboratory evidence of disease-specific drug synergism with irinotecan. A retrospective analysis of 34 heavily pretreated metastatic pancreatic cancer patients treated with G-FLIP combination (irinotecan dose = 80 mg/m^2) reported a partial response rate of 24%, disease stabilization rate of 21% and a median survival of 10.3 months. The regimen was well tolerated with most observed toxicities being grade 1–2 mucositis, nausea/vomiting, neurotoxicity, nephrotoxicity and diarrhea [1]. A review of 15 patients receiving the four-drug regimen as initial treatment of metastatic pancreatic cancer documented a 33% response rate [17]. Based on this encouraging clinical activity, a phase I dose-finding study was initiated to determine the maximum tolerated dose (MTD) of irinotecan in combination with fixed doses of gemcitabine, 5-FU/leucovorin (LV) and cisplatin in metastatic solid tumors.

Materials and methods

Patient selection

Patients with a histologically or cytologically confirmed diagnosis of a solid tumor refractory to conventional treatment or for which no standard therapy existed were eligible for this phase I study. Once MTD was defined, accrual was transitioned to the phase II trial and enrollment was limited to patients with metastatic pancreatic cancer. Other eligibility criteria included the following: age $\geq$ 18 years, Karnofsky performance status (KPS) of $\geq$ 60, life expectancy of at least 12 weeks; no chemotherapy, immunotherapy or radiotherapy for at least 4 weeks prior to entry into the study (6 weeks for nitrosureas or mitomycin C); no concurrent therapy including chemotherapy, immunotherapy, radiotherapy or any other investigational drug; no prior therapy with a topoisomerase I inhibitor; measurable or evaluable disease; adequate hematopoietic (absolute granulocyte count of $\geq$ 1500/mm^3 and platelet count $\geq 100 \times 10^9$/l), renal (creatinine of $\leq$ 1.5 mg/dl) and hepatic function (bilirubin $\leq$ 2.0 mg/dl); negative pregnancy test documented prior to study entry for premenopausal women; men and women who were fertile must have used adequate contraception. Exclusion criteria included patients with brain involvement or leptomeningeal disease; progressive sensory neuropathy or hearing loss; serious illnesses or medical conditions including uncontrolled diabetes, hypertension or arrhythmias, congestive heart failure or unstable angina, active infection, prior invasive malignancies within 5 years with an exception of curatively treated basal or squamous cell carcinoma of skin or carcinoma in situ of the cervix. A signed informed consent was obtained from all patients before study entry. The protocol had approval of a local IRB.

Dosage and drug administration

Therapy was administered in an outpatient setting every 2 weeks and consisted of gemcitabine 500 mg/m^2 i.v. in 100 cm^3 normal saline at 10 mg/m^2/min, followed by irinotecan (per dose escalation schema) in 500 cm^3 D5W over 90 min, followed by LV 300 mg in 50 cm^3 normal saline i.v. over 10 min, followed by 5-FU 400 mg/m^2 in 50 cm^3 normal saline over 10 min, followed by 5-FU 1500 mg/m^2 via an AIM pump (Ambulatory Infusion Manager; Abbott, Chicago, IL) over 24 h on day 1. On day 2, 24 h after the day 1 5-FU bolus, patients received 35 mg/m^2 of cisplatin in 50 cm^3 normal saline i.v. over 45 min.Prior to receiving cisplatin, mannitol 12.5 g was administered i.v. in 500 cm^3 D5 0.5 normal saline over 30 min and an additional 25 g of mannitol in 1000 cm^3 0.5 normal saline with 30 meq of potassium chloride (KCl) and magnesium sulfate 2 g was administered upon completion of cisplatin infusion. Cisplatin infusion was started once urine output reached at least 100 cm^3/h with 10–20 mg of lasix given immediately before cisplatin infusion. The starting irinotecan dose was 80 mg/m^2 and was escalated stepwise by 20 mg/m^2 increments in successive cohorts of three patients until the MTD was reached. Additional patients could be enrolled at a particular dose level to further evaluate toxic side-effects.

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At each dose level, the initial patient was observed for 4 weeks (2 biweekly cycles) prior to entry of subsequent patients. Subsequent patients at each dose level were evaluated weekly for at least 2 weeks before accrual at the next dose level could take place. Weekly evaluations consisted of a toxicity check, physical examination and laboratory evaluation (complete blood count, chemistry profile including serum creatinine, electrolytes and hepatic function).

Assessment of toxicity and response

Dose-limiting toxicity (DLT) was defined as grade 3 or greater non-hematologic toxicity (including nausea/vomiting despite aggressive antiemetic therapy) or inability of the patient to take 75% or more of planned chemotherapy. If DLT developed in one of three patients, then three additional patients were to be enrolled at that dose level. If two or three out of three initial patients or more than one out of three additional patients treated at a dose level developed DLT, dose escalation was to be stopped. MTD was defined as the dose level below the dose that produced unacceptable toxicity. Additional patients were to be enrolled at the MTD in the phase II portion of the study to further characterize disease specific efficacy and toxicity.

Toxicity was graded using the National Cancer Institute Common Toxicity Grading Criteria (version 2.0). Drug specific dose adjustments were made prior to subsequent cycles in case of toxicity. Cisplatin dose was decreased by 25% for neutropenic fever, platelet counts $\leq 100\,000/m^3$ or for grade 1–2 persistent sensory neuropathy. Cisplatin was discontinued for grade 3–4 sensory neuropathy. Cisplatin dose was reduced to $20\,mg/m^2$ and administration changed to a continuous infusion if serum creatinine rose to 1.5–3 mg/dl. Infusional 5-FU dose was reduced by 25% for grade 3–4 stomatitis or grade 2 or greater hand–foot syndrome. Irinotecan-associated acute or delayed diarrhea was treated symptomatically with atropine and loperamide, and irinotecan dose was reduced by 25% for grade 3–4 diarrhea.

Tumor responses were evaluated every 8 weeks by objective, two-dimensional measurements of evaluable tumors along the longest diameter according to RECIST criteria, employing imaging studies such as computed tomography scans or magnetic resonance imaging. A complete response (CR) was defined as the disappearance of all measurable and evaluable disease for at least 4 weeks without appearance of new lesions. A partial response (PR) was defined as at least 30% decrease in the sum of longest diameter of all measurable lesions from baseline without appearance of new lesions. Progressive disease (PD) corresponded to at least 20% increase in the sum of the longest diameter of the measurable lesions or the appearance of new lesions. Stable disease (SD) was defined as insufficient decrease in tumor to qualify for a PR or insufficient increase in size to qualify for PD. Toxicity was evaluated in patients who received at least two G-FLIP cycles. Patients who achieved a CR could continue treatment for up to 6 months beyond the documentation of complete response. All patients with PR or with SD were continued until documentation of disease progression. Patients were withdrawn from the therapy in case of progressive disease, patient refusal, physician's preference or development of any toxicity that would preclude further therapy. Response durations were measured from the time of documented radiographic response to the first observation of progressive disease. Time to disease progression was measured from the start of the treatment to first documentation of disease progression.

Study design

This study was designed as a phase I–II, open label, non-randomized dose finding study. In the phase I study, the first three eligible patients were assigned to receive treatment at dose level 0. At least three patients were studied for 28 days at each dose level before starting additional patients on escalated doses of irinotecan. In the event that the MTD was less than irinotecan $100\,mg/m^2$ biweekly the dose of cisplatin was to be reduced to $30\,mg/m^2$ biweekly and irinotecan dose escalation would resume at one dose level below the previous MTD. When the MTD of irinotecan was determined, accrual was to be transitioned to the phase II aspect of the trial. Accrual to the phase II study was limited to patients with metastatic pancreatic cancer in order to fully define disease specific efficacy as well as to further characterize the toxicity profile of this novel regimen.

Results

Patient characteristics

Twenty-three patients were enrolled in the phase I study between March 2002 and February 2003. Patient characteristics are illustrated in Table 1. Thirteen men and 10 women with a median age of 63 years and median KPS of 80 were enrolled. Ten patients (43%) received prior treatment: two patients received surgery alone, two patients received either chemotherapy alone or in combination with surgery, one patient received chemotherapy and radiation, and five patients received surgery, chemotherapy and radiation. All 23 patients enrolled into the phase I study were assessable for toxicity after completing a minimum 2 cycles of therapy. Eighteen of these patients were evaluable for response. Five patients were withdrawn from the trial prior to response evaluation for the following reasons: one patient had severe asthenia necessitating withdrawal from study after 1 cycle and four patients had early disease progression after 1 or 2 cycles of G-FLIP. The initial seven patients with metastatic pancreatic cancer enrolled in the phase II portion of this trial are evaluable for

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toxicity. Four were men and three women; median age was 54. Five of these patients previously were untreated and two had received prior gemcitabine-based regimens.

Treatment administration and toxicity

A total of 134 cycles were administered and each patient received a median of 8 cycles (1–16). From a starting dose of 80 mg/m^2 of irinotecan, dose escalation proceeded until dose level 4 (160 mg/m^2). Hematologic and non-hematologic toxicities are summarized in Table 2. No DLTs were identified at dose level 0, and one out of three patients at dose level 1 required hospitalization for nausea and vomiting. Subsequently, three additional patients were enrolled at dose level 1 without additional DLTs. All patients at dose level 2 tolerated therapy without DLTs. Six patients initially were enrolled at dose level 3, per protocol, permitting accrual of additional patients at a particular dose level to further evaluate toxic side-effects. One of these six patients developed grade 3 nausea and vomiting requiring hospitalization and i.v. hydration. Therefore, an additional three patients were enrolled at dose level 3, two of whom developed refractory grade 3–4 nausea/vomiting requiring hospitalization in spite of aggressive antiemetic therapy. At the principal investigator's discretion, one patient was entered at dose level 4 before the DLT and MTD were determined. Grade 2 nausea and vomiting occurred at that dose. In light of the above events, dose level 2 was identified as the MTD. Per study design, accrual was then transitioned to the phase II part of the study. The initial seven patients enrolled onto the phase II trial were evaluable for toxicity and none experienced DLT.

Outside of the DLT, therapy was well tolerated and grade 3–4 toxicities per patient were largely hematological, and consisted of anemia (6%), thrombocytopenia (3%), neutropenia (16%) and neutropenic fever (10%). Hematologic toxicities were consistent throughout all dose levels. Grade 3–4 thrombosis occurred in seven patients (23%), so the protocol was amended with prophylactic coumadin 1 mg daily recommended. Grade 3–4 non-hematologic toxicities were limited to nausea/vomiting and fatigue in four (13%) and seven patients (23%), respectively. Grade 3–4 nausea/vomiting was observed in one patient at dose level 1 and three patients at dose level 3. Other non-hematologic toxicities were mainly grade 1–2 nausea/vomiting (60%), diarrhea (40%), constipation (16%) and fatigue (30%). Grade 1–2 ototoxicity and neurotoxicity was seen in two patients. Two patients developed cisplatin hypersensitivity, during cycles 5 and 11, respectively. These hypersensitivity reactions were characterized by intense chest pressure and light-headedness in one patient, and chest pressure and diffuse skin erythema in the other patient. Both of these reactions

Table 1 Phase I patient characteristics

Characteristic	No. of patients	%
No. entered	23	100
Sex		
male	13	56
female	10	44
Age (years)		
median	63	
range	44–78	
Performance status (KPS)		
60	1	4
70	3	13
80	11	48
90	6	26
100	2	8
Prior therapy[a]		
none	13	56.5
surgery alone	2	8.6
surgery, chemotherapy and radiation	5	21.7
chemotherapy and radiation	1	4
surgery and chemotherapy	1	4
chemotherapy alone	1	4
Primary site		
pancreas	11	47
gallbladder	5	21
hepatocellular	1	4
head and neck	3	13
gastric	1	4
melanoma	1	4
breast	1	4

[a]Other prior treatments included interferon in one patient.

Table 2 Toxic side-effects (event per patient)

	Dose level 0 (n=4)		Dose level 1 (n=6)		Dose level 2 (n=10)		Dose level 3 (n=9)	
	Grade 3–4	Grade 1–2	Grade 3–4	Grade 1–2	Grade 3–4	Grade 1–2	Grade 3–4	Grade 1–2
Hematological								
neutropenia	0	0	3	2	1	0	1	0
thrombocytopenia	0	0	1	1	0	1	0	0
anemia	1	NR	1	NR	0	NR	0	NR
neutropenic fever	0	NA	2	NA	0	NA	1	NA
thromboembolism	1	NA	4	NA	1	0	1	NA
Non-hematological								
neuropathy	0	0	0	1	0	1	0	0
ototoxicity	0	0	0	2	0	0	0	0
constipation	0	0	0	3	0	1	0	1
nausea/vomiting	0	2	1	5	0	7	3	4
fatigue	0	1	2	2	3	3	2	3
diarrhea	0	0	0	2	0	5	0	5

NR, not reported; NA, not applicable.

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occurred within the first minute of cisplatin infusion, and recurred despite appropriate premedication with steroids and diphenhydramine upon rechallenge. Cisplatin was, therefore, discontinued in these two patients.

Tumor response

Eighteen of 23 patients were evaluable for response. One patient with locally advanced pancreatic cancer attained a radiological CR after 4 cycles of therapy and remained in CR following 8 cycles. Eight patients (44%) had PRs: three patients with gallbladder cancer, two with pancreatic cancer, one patient with adriamycin/taxane refractory breast cancer, and two patients with platinum/taxane refractory head and neck cancer (tongue and sinus). Seven patients (39%) attained disease stabilization: five pancreatic, one hepatocellular and one gastric cancer. Median time to disease progression among all 23 patients was 20.5 weeks (range 4–37 weeks).

Discussion

The chemotherapy agents in G-FLIP all have proven single-agent activity in a wide variety of malignancies. A clear rationale exists for combining these drugs based on preclinical and clinical data. Three drug and four drug regimens have been evaluated in pancreatic cancer. A retrospective analysis of 49 patients with advanced pancreatic cancer treated with gemcitabine, 5-FU/LV and cisplatin reported a median survival of 10.6 months and a 1-year survival of 46%. Grade 3–4 toxicities were hematological [18]. A four-drug combination of cisplatin and epirubicin on day 1, gemcitabine on days 1and 8, and 5-FU continuous infusion daily for 28 days given to patients 49 with advanced pancreatic cancer (43 patients had metastatic disease) reported an objective response rate of 58% and clinical benefit in 22 (78%) of 28 assessable patients. The median survival was 10 months in the intent-to-treat population [19].

Table 3 illustrates the increasing response rates and overall survival associated with combination chemotherapy, providing the rationale for multidrug combinations in pancreatic cancer. Most phase II trials of gemcitabine-based doublets have reported median survivals of 7.5–8.5 months, but these modest advances did not withstand analysis in phase III trials [20–23]. However, median survivals of 10–11 months are consistently reported in phase II trials of three- and four-drug regimens. These survival outcomes may represent a threshold that will translate into clinically meaningful advances in phase III testing.

This trial was a phase I dose escalation study designed to determine the MTD of irinotecan in combination with fixed doses of gemcitabine, 5-FU/LV and cisplatin given as an outpatient regimen on an every-2-weeks schedule in patients with advanced solid tumors. The DLT was 5-HT_3 antagonist-refractory nausea and vomiting occurring at irinotecan dose level 3 (irinotecan = 140 mg/m^2). Cisplatin was dose reduced by 25% in only two patients due to delayed emesis. The subsequent FDA approval of aprepitant for delayed chemotherapy-associated nausea and vomiting may improve the tolerability of this regimen. Other non-hematologic toxicities were mild. Except at dose level 0 (irinotecan 80 mg/m^2), grade 3–4 neutropenia occurred at a rate of 33% of patients/dose level. Grade 3 anemia requiring transfusions occurred in 9% of patients. Hematopoietic colony stimulating factors like filgrastim or sargramostim and erythropoietin were administered according to the indications set forth by American Society of Clinical Oncology [24]. Grade 3 thrombocytopenia was seen in two patients and was self limiting without bleeding complications or need for platelet transfusion. Hypersensitivity reactions to i.v. cisplatin are a rare, but life-threatening, complication that may occur even in patients who have received prior treatment with cisplatin. The appearance of hypersensitivity reactions was reported in patients treated with concomitant pelvic radiation and weekly i.v. cisplatin for gynecologic malignancies [25].

The 31% incidence of thrombotic events with five patients developing deep venous thrombosis and two patients presenting with uncomplicated pulmonary embolism is notable. All patients with thrombotic events were treated with therapeutic doses of low-molecular-weight heparin. Hypercoagulability in cancer patients

Table 3 Phase II reports of two-, three- and four-drug regimens for locally advanced and metastatic pancreatic cancer

Treatment	No. patients	LA/M (%)	RR (%)	Median PFS (months)	Median OS (months)	Reference
Gem (phase III)	163		5.6	2.2	5.4	20
Gem/5-FU bolus	164	10/90	6.9	3.4	6.7	
Gem 10 mg/m^2/min/5-FU bolus	34	24/76	17	3.7	5.7	29
Gem + 5-FU bolus and infusion (FOLFUGEM)	62	35/65	26	4.8	9.0	30
Gem + cisplatin biweekly	35	15/85	11.5	4.3	8.3	31
Gem + oxaliplatin	34	0/100	31	4.1	8.7	32
Gem + irinotecan	45	28/72	20	2.8	5.7	14
Gem/5-FU/cisplatin	49	0/100	16.3	2.1	10.6	18
Gem/5-FU/irinotecan/cisplatin	34	0/100	23.5	2.5	10.3	1
Gem/cisplatin/epirubicin/5-FU	49	0/100	58	7.5	10	19
Iriotecan/oxaliplatin/5-FU	23	0/100	50	7.5	NS	33

LA, locally advanced disease; M, metastatic disease; Gem, gemcitabine; NS, not specified.

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remains a complex and poorly understood problem. Activation of factor X, increased fibrinogen and platelet catabolism, decreased protein C, S and antithrombin III as well as direct generation of thrombin have been implicated in this process [26]. In one series from the UK, the estimated prevalence of venous thromboembolism in advanced pancreatic cancer patients was over 50% [27]. It is not known to what extent irinotecan, 5-FU, LV and drug administration schedule contribute to the rate of thromboembolism observed in this trial. During the panel review of CPT-11 trials in colorectal cancer, Rothenberg *et al.* reported a 16% incidence of thromboembolic events with a combination of irinotecan, infusional 5-FU and LV compared to 9% with infusional 5-FU and LV [28]. All subsequently enrolled patients were started on low-dose coumadin for thromboprophylaxis.

Significant antitumor activity in a wide variety of solid tumors including pancreas, gall bladder, hepatocellular, head and neck, and anthracycline/taxane refractory breast cancer was observed. In summary, G-FLIP is a well-tolerated regimen, administered in an outpatient setting on a biweekly basis. This regimen appears to be particularly active in pancreatic and gallbladder cancers. The DLT was intractable nausea and vomiting requiring hospitalization and i.v. fluid support. The recommended dose of irinotecan for phase II study is 120 mg/m^2 in combination with fixed doses of gemcitabine, 5-FU/LV and cisplatin. Phase II testing in pancreatic cancer patients is ongoing.

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Review article: Angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy

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For many years the emphasis in cancer research and therapy has been on the difference in proliferation rate that results from malignant transformation. The original concept that tumour growth was uncontrolled is now recognized to be an oversimplification. The cells in different tumours have characteristic rates of proliferation, which are only translated into an increase in mass if the rate of cell production and cell loss are unequal. In normal tissues natural cell loss is balanced exactly by cell production but in tumours both the mechanisms of cell loss and its extent are altered. With the new technologies of molecular biology allowing a more detailed study of deoxyribonucleic acid (DNA) changes in normal and malignant cells, the focus of attention is now on oncogenes, growth factors and their receptors. This may lead to a deeper understanding of the reasons for the unbalanced growth but is not yet having much impact on curative treatment strategies.

For any tumour mass to grow to macroscopic dimensions it must, of necessity, induce the surrounding normal tissue either to share its nutrient supply or to provide an alternative supply. Thus the mark of a successful malignant transformation involves the ability to induce an expansion of the vascular network to provide all the nutrients needed for cellular proliferation. This process of "angiogenesis" has been studied intensively by Folkman and colleagues for about two decades, with inhibitors of the angiogenic stimulus often being detected using tumour implants into the chorioallantoic membrane of the chick egg or the cornea of the rabbit's eye. Many of the substances detected in this way have then been shown to influence growth of subcutaneously transplanted tumours. A correspondingly large number of substances have been identified which will interfere with the process of angiogenesis (Folkman et al, 1971, 1987, 1989).

An alternative strategy which also identifies blood vessels as the target is aimed at damaging the newly formed vessels with the intention of causing blockage, haemorrhage, thrombosis or collapse of these vessels, leading to ischaemic or haemorrhagic necrosis. In this way, by occluding individual capillaries many thousands of tumour cells can be killed as a result of starvation. The concept of targeting the stromal support of the tumour requires an understanding of the pathophysiology of vascular networks, the factors that control vessel tone, permeability, cardiac output, tissue distribution, blood viscosity, coagulation, tissue specific inflammation, endothelial-leukocyte adhesion and cytokine-mediated immunity. For this approach we need to study the tumour with its host as a whole system and not focus on features of the individual transformed malignant cells. Indeed it is the "activated" normal (vascular) cells that will be the target of this approach. The fact that activation has resulted from a stimulus provided by malignant cells may be of secondary importance. Some, though not necessarily all, features of vessels in tumours may be shared with the neovasculature induced in other normal or non-malignant pathophysiologies, *e.g.* in arthritic or diabetic angiopathies, placenta and wound healing. However, the quality of the blood flowing through the long vessel loops in tumours is often poor in nutrients and high in catabolites. Furthermore, the cells adjacent to capillaries may influence endothelial cell function, *e.g.* by cytokine activation of coagulation precursors.

Many existing forms of therapy are influenced by the poor vasculature that is associated with solid tumours. In the past the emphasis has been on the problems that this creates for the therapist, but over the last decade it has emerged that for many treatments this poor neovasculature may actually be the basis for the beneficial therapeutic difference between tumour and normal tissues (Denekamp, 1982, 1990).

Cell kinetic studies

Most tumours invade normal tissues but discrete solid masses only appear if the tumour cells can force the adjacent vascular network to expand to supply their needs. The production of factors that stimulate angiogenesis has been studied for many years. Initially, lactic acid and other by-products of anaerobic glycolysis were considered to be the angiogenic stimulus. In the early seventies, however, Folkman and his colleagues (1971)

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Table I. Substances with effects on vascular proliferation

Angiogenic activity	Anti-angiogenic activity
aFGF	TGFβ
bFGF	α Interferon
ESAF	TNF γ
TNF	TIMP 1
TAF	TIMP 2
Prostaglandins	Protamine
PGE1, PGE2	Cartilage extract
Angiotropin	Hydrocortisone
Angiogenin	Heparin
Angiotensin	Retinoids
Heparin	Methotrexate
Histamine	Mitoxantrone
Lactic acid	Pencillamine
Leukotriene B4	Fumagillol

For details and references, see McLaughlin, 1991 and Maione & Sharpe, 1990.

showed that crude extracts of a tumour had mitogenic properties for endothelial cells *in vitro* and in other *in vivo* models. Their crude extract was termed Tumour Angiogenesis Factor (TAF). They have gone on to study the inhibitors of TAF and the cofactors that are needed for angiogenesis and have produced an impressive stream of papers over the past 20 years, *e.g.* Folkman et al, 1987, 1989; Ingber et al, 1990. Many other groups have also entered this field and have extracted angiogenic factors from a variety of tumours, normal tissues or cultured cells. Some factors have been identified at the molecular level and produced as a cloned product, *e.g.* angiogenin, but others are still a chemical and biological mystery (Folkman & Klaagsbrun, 1987; Maione & Sharpe, 1990). The factors cover a wide spectrum of molecular weights, from large molecules such as the Fibroblast Growth Factors (MW ~ 20,000 d) to the small Endothelial Cell Stimulating Angiogenic Factors (MW ~ 500 d). These angiogenic agents may have independent actions or may be cofactors in a complex chain of events. A growing panel of anti-angiogenic agents is also being identified, with an equally wide spectrum of molecular structures. It is now evident that angiogenesis is a complex response and it is perhaps not surprising that it can be blocked at many levels (Table I).

Expansion of the vascular network to provide the nutritional requirements of the tumour cells is a complex process, with the first step being the induction of buds from pre-existing mature vessels, usually from venules on the low pressure side of the circulation. The formation of a capillary bud involves at least three discrete events: localized dissolution of the basement membrane, migration of the endothelial cells towards the angiogenic stimulus and cell proliferation to provide the necessary extra endothelial cells. It now seems likely that most of the identified angiogenic factors cause one or two, but seldom all three, of these responses. When the vessels have become incorporated in the tumour mass they must then elongate to keep pace with the expanding mass. For the network to be effective two further processes, branching (by further budding of the new vessels) and anastomosis of the buds with adjacent vessels, are necessary. This process of angiogenesis requires proliferation of the constituent cells of the vessel wall which in tumours are mainly endothelial cells. Studies with tritiated thymidine have been performed on blood vessels in a wide range of tumours and normal tissues in mice, and in a much more limited series of human tumours (Denekamp & Hobson, 1982; Hobson & Denekamp, 1984; Denekamp, 1986). These studies have shown that normal vessel endothelium is remarkably stable, with a thymidine labelling index of only 0.01–1.0% (median value 0.2%). By contrast, in tumour vessels the values range from 1–32%, with a median value of 9%. Table II shows the potential rate at which the vascular network could double in some tumours if the duration of DNA synthesis was 10 or 15 h. There is a very different rate of cell production and a remarkably clear distinction between all normal tissues and all solid tumours. Such a consistent difference is rarely identified in cancer research (Denekamp & Hobson, 1982).

Replacement of cells lost by wear and tear in mature vessels occurs very rarely. Production of endothelial cells, however, is very fast in tumours. The potential doubling time is months to years for normal vessel endothelium and less than a week for many tumour vessels. When this huge difference was first recognized we postulated that it might be possible to develop antibody-mediated therapies using antibodies raised against proliferating endothelial cell markers (Denekamp, 1982). That is still an active research area (Hagermeier et al, 1986; Schlingemann et al, 1990). Subsequently, however, we have realized that there may be many other characteristics of the neovasculature that will also allow agents to have a differential effect against the vessels including permeability, coagulation control and adhesion molecule expression in tumours and normal tissues (*e.g.* Murray 1991).

Tumour vessels form a chaotic disorganized network with tortuous thin-walled vessels traversing the tumour mass. It is commonly believed that the rare vessels seen in a tumour with a well developed multilayered wall, and with muscular and neural elements, are those that have been parasitized and engulfed by the expanding tumour mass. The branching pattern of the fragile neovasuclar network is inadequate and broad sinusoidal vessels and blunt ended, presumably non-functional, vessels can be seen in corrosion casts. Intercapillary distances become so large that dead cells are sometimes visible between the sleeves or cords of tumour cells that surround each capillary. Such tumour cords were demonstrated many years ago in human tumours using haematoxylin and eosin stains. New staining technologies, involving the bioreductive properties of nitroimidazoles, now allow the biochemically hypoxic cells to be identified at the boundary between the viable tissue and

Table II. Uptake of tritiated thymidine into endothelial cells in rodent tumour blood vessles

Tumour	L.I %	Potential doubling time (days) if Ts = 10 h	Potential doubling time (days) if Ts = 15 h
Mammary carcinoma C3H	11.4	2.9	4.4
Mammary carcinoma C3H	14.0	2.4	3.6
Mammary carcinoma RH	4.5	7.4	11.1
Mammary carcinoma KHH	17.7	1.9	2.8
Mammary carcinoma KHU	17.9	1.9	2.8
Anaplastic sarcoma RIB5	32.3	1.0	1.5
Fibrosarcoma KHJ	18.0	1.8	2.8
Lymphoma KHAA	3.6	9.3	13.9
Rhabdomyosarcoma KHKK	16.5	2.0	3.0
Fibrosarcoma KHTD	16.7	2.0	3.0
Mammary adenocarcinoma TB	10.2	3.3	4.9
Mammary adenocarcinoma AD	14.1	2.4	3.6
Mammary carcinoma KHLL	0.95	3.5	5.3
Mammary adenocarcinoma BAC	9.0	3.7	5.6
Squamous carcinoma SQ D	7.1	4.7	7.1
Mammary adenocarcinoma KHLI	7.7	4.3	6.4
Mammary adenocarcinoma KHHH	8.0	4.2	6.3
Hepatoma KHI	10.5	3.2	4.8
Mammary adenocarcinoma RHf	6.6	5.0	7.5
Fibrosarcoma SA sf	3.9	8.5	12.8

Assumes a correction factor of 0.8 for λ in equation $T_{pot} = \lambda$ Ts/LI.

necrosis (Hodgkiss et al, 1991). It seems likely that oxygen is the critical nutrient that is depleted in respiring cell masses, leading to necrosis when it runs out. A further characteristic of tumour vessels is their cyclic closure which may last for minutes or for several hours, creating acutely and/or intermittently hypoxic cells (Brown, 1979; Trotter et al, 1989). The reason for this is unclear but it may relate to vessel blockade by leukocytes or fluctuations in interstitial pressure such that the small difference in intravascular: interstitial pressure is temporarily reversed and the vessel collapses until the pressure differential is restored.

Anti-angiogenesis *versus* anti-neovasculature

The concept of anti-angiogenic therapy relates to interfering with the stimulating substances that cause new vessel formation. This should be important in preventing establishement of small solid tumours or in preventing metastases. By contrast, vascular targeting, *i.e.* attacking the existing neovasculature, is likely to be most effective on large tumours, where the vasculature is already compromised. The target may be the proliferating immature endothelial cells of the vessel wall or may be related to the lack of innervation and musculature, the very different characteristics of the blood within these vessels or the lack of lymphatic drainage. The features of the vasculature that may allow these two different vascular mediated strategies to be utilized as the Achilles heel for tumour therapy are compared in Figure 1.

The anti-angiogenic strategies should prevent development of new vessels and/or the expansion of a neovascular network. However there is no logical reason why this should lead to tumour regression if no damage is done to the pre-existing stromal cells or to the tumour cells themselves. The models (*e.g.* chorio-allantoic membrane and the rabbit cornea) in which regression of newly formed vessels occurs if the angiogenic stimulus is removed do not necessarily reflect the situation in an established tumour, where the very existence of metabolizing cells may be adequate to provide a continuing need for the vessels already produced to remain if the stimulus for further angiogenesis is blocked.

Vascular targeting, by contrast, involves any metabolic or patho-physiologic aspects of the tumour vessel that can be used to induce blockade, collapse, stasis, haemorrhage or thrombosis. If a capillary, or a sector of the capillary bed, fails many thousands of dependent tumour cells will die of nutrient deprivation. Indeed, one of the most characteristic features of vascular-induced injury is the appearance of massive patchy necrosis with the individual capillaries that are still functional being surrounded by a normal cuff of 6–10 cell layers. Since such vascular collapse can occur in large tumours as a normal component of the growth pattern of a tumour it may be necessary to use markers to identify whether the necrosis was induced by a vascular-mediated treatment. An appropriate marker in the clinic might be to prelabel the tumour cells with bromodeoxyuridine (BUdR) which is only taken up by viable proliferating cells and which can be visualized with a monoclonal antibody. If this is given shortly before a putative vascular-mediated therapy, BUdR-labelled DNA should be detectable 12–24 h later in the necrotic zones. Alternatively, label-

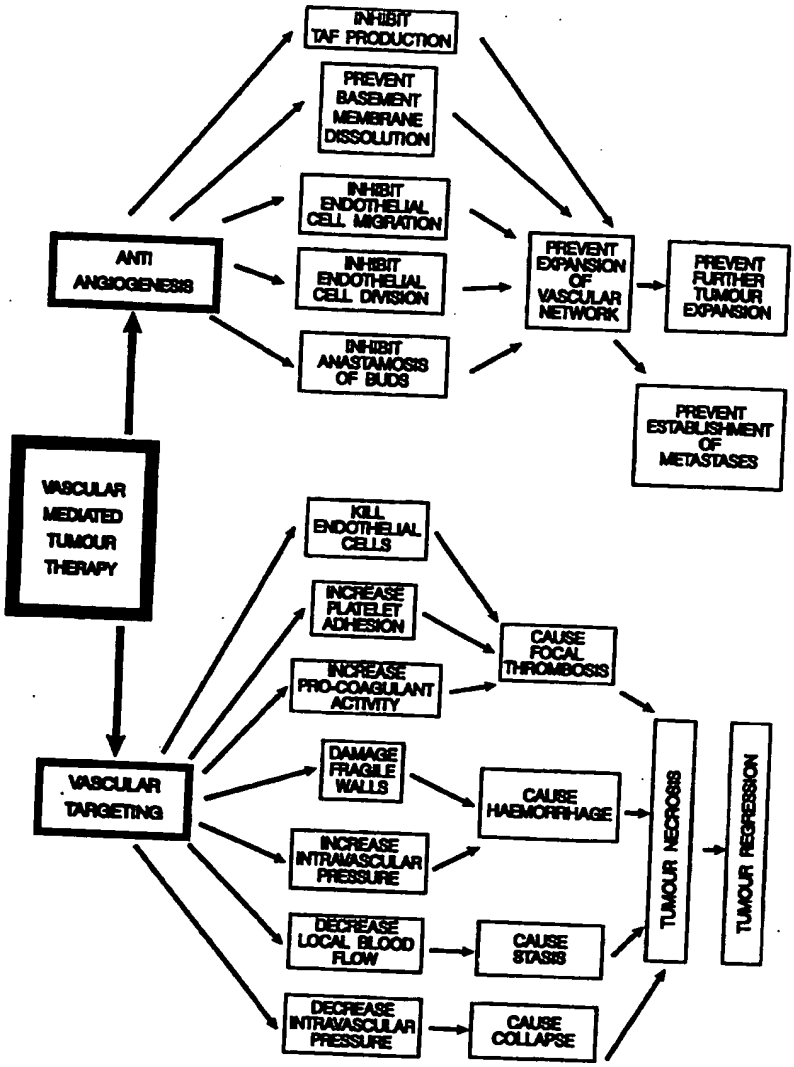

Figure 1. Diagram to illustrate the differences between the concepts and the likely outcome of anti-angiogenic strategies and those designed to produce ischaemic or haemorrhagic necrosis by vascular targeting.

ling samples of erythrocytes with two separate isotopes would allow the detection of perfusion through vessels within necrotic regions and determination of whether perfusion of some areas had ceased between the delivery of the first and second label (*e.g.* before and after the vascular targeted therapy).

Radiotherapy and modifiers of radiation sensitivity

The excessive spacing of capillaries which exceeds the oxygen diffusion gradient gives rise to the corded structure, first described by Thomlinson & Gray in 1955, with hypoxic cells occurring at 100–150 μm from the central vessels and finally giving rise to necrosis.

The emphasis in radiobiology has for decades been on the radioresistance problems posed to the therapist by this inadequate vasculature (*e.g.* Gray et al, 1953; Adams et al, 1978). Virtually no attention has been paid to the concept that deliberate induction of vascular collapse would lead to an enhancement of the hypoxia-mediated killing that is giving the characteristic pattern of necrosis. Indeed after irradiation a process of "super-vascularization" has been described in shrinking tumours where the ratio of vessels to tumour cells appears to increase as the tumour cells are killed and resorbed. This does not necessarily mean that extra vessels have been formed, but that perhaps vessels are not immediately resorbed if the dependent tumour cells diminish in number. X rays have, however, also been shown to cause vascular collapse with vessel occlusion by thrombosis being described when the tumours start to regrow. Detailed morphometric studies have demonstrated that the occlusion is mainly in the narrower 10 μm nutritive capillaries, with only small effects in vessels of 20–30 μm diameter (Solesvik et al, 1984). Indeed, the radiation damage to subcutaneous vascula-

ture can be demonstrated by implanting untreated tumour cells into a previously irradiated area and monitoring the extent to which tumour growth is retarded compared with tumours implanted into untreated skin. This is described as the Tumour Bed Effect and probably also occurs in recurrent tumours after inadequate therapy. Because the vascular damage induced by radiation is small and is mainly detetectable as the tumours regrow it seems unlikely to account for most of the observed tumour cell kill that is needed to lead to local control.

The use in the sixties and seventies of new approaches, *e.g.* of X rays with hyperbaric oxygen or of high linear energy transfer (LET) radiations such as neutrons, were mainly based on the concept of hypoxic radioresistance which could be abolished by increasing the supply of oxygen or delivering lesions that were so severe that chemical redox repair was irrelevant. In the seventies these gave way to oxygen-mimetic chemical radiosensitizers which preferentially sensitize tumours because of their hypoxic cells. These are usually redox compounds, believed to interact with radiation-induced free radicals, in a manner similar to oxygen. If oxygen is already present they are ineffective. Misonidazole (a 2-nitroimidazole) is the prime example of a tumour radiosensitizer whose radiosensitizing effect is clearly limited to hypoxic cells. Animal data showed significant sensitization of tumours with little or no effect on normal tissues if miso or other nitroimidazoles and large single radiation doses were used (Adams et al, 1978). This benefit diminished with fractionation because of a natural process of reoxygenation of the hypoxic cells. Several different compounds including misonidazole, metronidazole, pimonidazole and nimorazole have all been tested in the clinic (Dische, 1991). Although few individual clinical studies with oxygen or chemical sensitizers have shown a significant gain, a recent meta-analysis of all 72 clinical studies indicates that benefit has emerged and that hypoxia does limit the success of conventional radiotherapy (Overgaard, personal communication). Recently a new approach to the hypoxic problem of radioresistance has appeared in the form of ARCON. This aims to combine Accelerated schedules with high Oxygen tensions (but at nomobaric pressures), Carbon dioxide and Nicotinamide. This multifactorial approach (Figure 2) aims to overcome resistance resulting from proliferation during treatment, to sensitize diffusion-induced and perfusion-limited intermittently hypoxic cells and to avoid the physiological feedback mechanisms that mitigate against oxygen toxicity, which may have led to vasoconstriction and reduced respiration rates with hyperbaric oxygen (Horsman et al, 1987; Kjellen et al, 1991; Rojas et al, 1992). Studies are also in progress to supplement this approach with assays for intrinsic radiosensitivity (of both tumour and normal cells) and of topical radioprotectors, *e.g.* WR2721 which may allow a slight dose escalation. The main focus in therapy-oriented radiobiology today is on multifactorial approaches and individualization of treatment.

Biochemical hypoxia as an aid in therapy

Miso was developed on basic physio–chemical principles as an electron acceptor which would allow fixation of free radical damage. It was therefore completely surprising when *in vitro* studies showed that it could be metabolized in hypoxic cells to a cytotoxic product in the absence of radiation (Brown, 1977). This was one of the earliest clear demonstrations that tumour hypoxia could give the cancer therapist an edge by converting a prodrug to a toxic metabolite. Bioreduction can occur in both oxic and hypoxic cells but the toxic product is detoxified in the presence of oxygen resulting in a futile cycle (Wardman, 1992). This has led to an expanding interest in the development of other bioreductive drugs, which should be metabolized to a toxic product only within the hypoxic cells that exist in solid tumours. Mitomycin C is an existing cytotoxic drug in the clinic which also was recognized in the seventies to have a component of its effect mediated by bioreductive activation since it is about twice as toxic to hypoxic *versus* oxic cells (Kennedy et al, 1980). The unexpected action of nitroimidazoles as bioreductive drugs has encouraged the development of a tumour-specific drug design approach based on the hypoxia in tumours rather than on malignant *versus* normal cell genetic differences. Drugs have now been developed with a huge difference in their effect on hypoxic *versus* oxic cells, *e.g.* a 50–100 fold lower concentration needed to cause hypoxic cell death (Stratford et al, 1992).

A further unexpected "autocatalysis of bioreduction" was found when tumour blood flow was also shown to be reduced by high doses of miso. When miso and the antimetabolite melphalan were used in combination an increased therapeutic benefit was observed which was originally attributed to independent subpopulations responding, *i.e.* the cycling cells close to vessels being killed by the melphalan and the distant hypoxic cells by the bioreductive activation of miso (Randhawa et al, 1984). However, a miso induced reduction in blood flow was shown subsequently which required a re-evaluation of the mechanism (Murray et al, 1987). It now seems likely that the enhanced cytotoxicity of these agents when they are combined may not be simply the result of effects on different subpopulations but may also involve pharmacokinetic changes (trapping of the melphalan within the tumour when blood flow ceases), increased conversion of the prodrug (miso) to the toxic metabolite as the degree of hypoxia increases, and even vascular-mediated ischaemic necrosis (Figure 3). These three vascular-mediated effects were completely unexpected when miso was being developed as a radiosensitizer. It is becoming increasingly apparent that other agents, developed for quite different reasons, may also have considerable effects on the tumour blood supply, *e.g.* the haemoglobin disocciation curve modifer BW12C (Honess et al, 1991). Furthermore, the reduction in blood flow induced by nitroimidazoles may be the reason for the totally unexpected detrimental effect recently observed with pimonidazole as a radiosensitizer in the clinic (Dische, 1991).

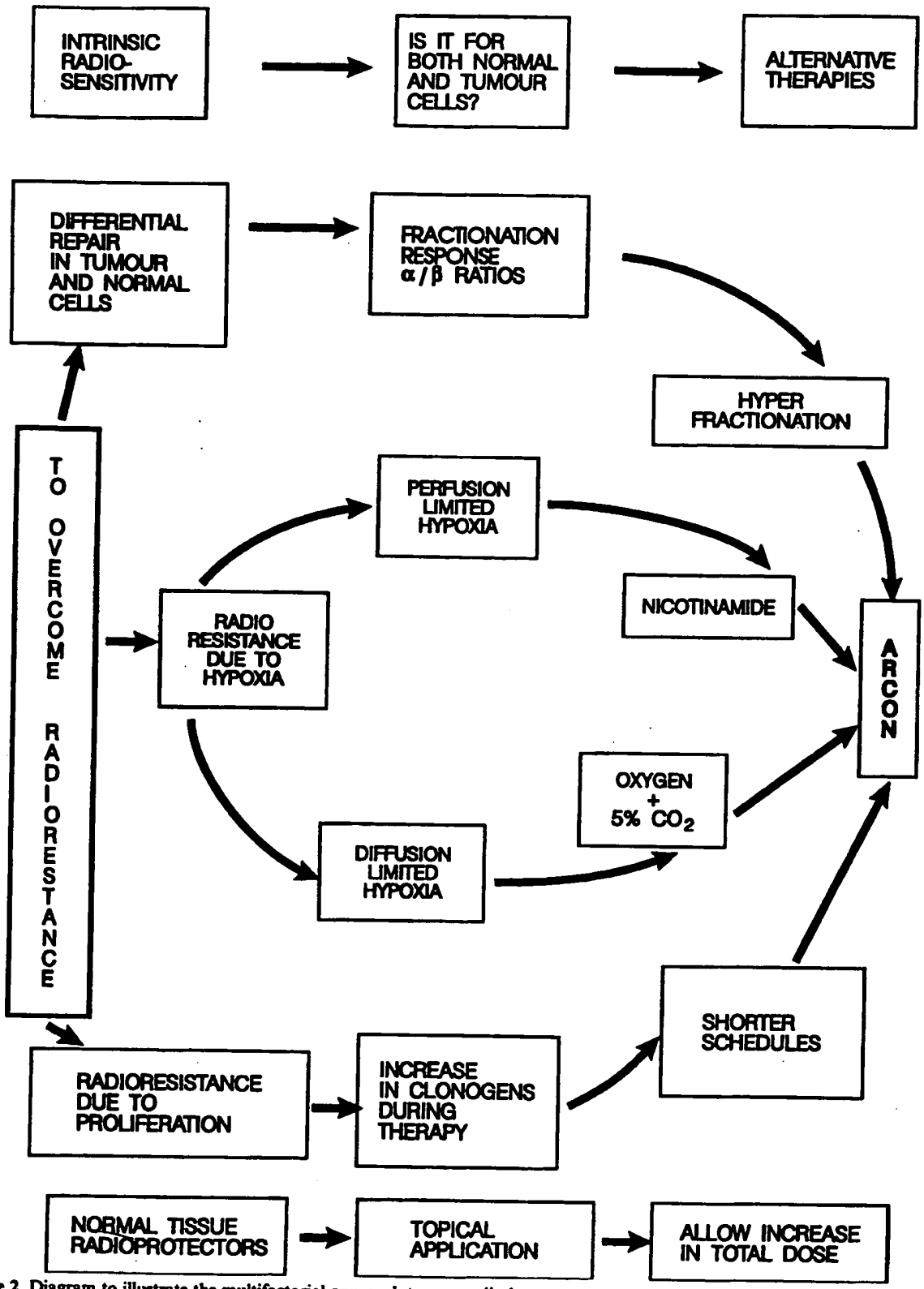

Figure 2. Diagram to illustrate the multifactorial approach to new radiotherapy protocols that allows several different aspects of radiobiological research to be integrated into a single treatment.

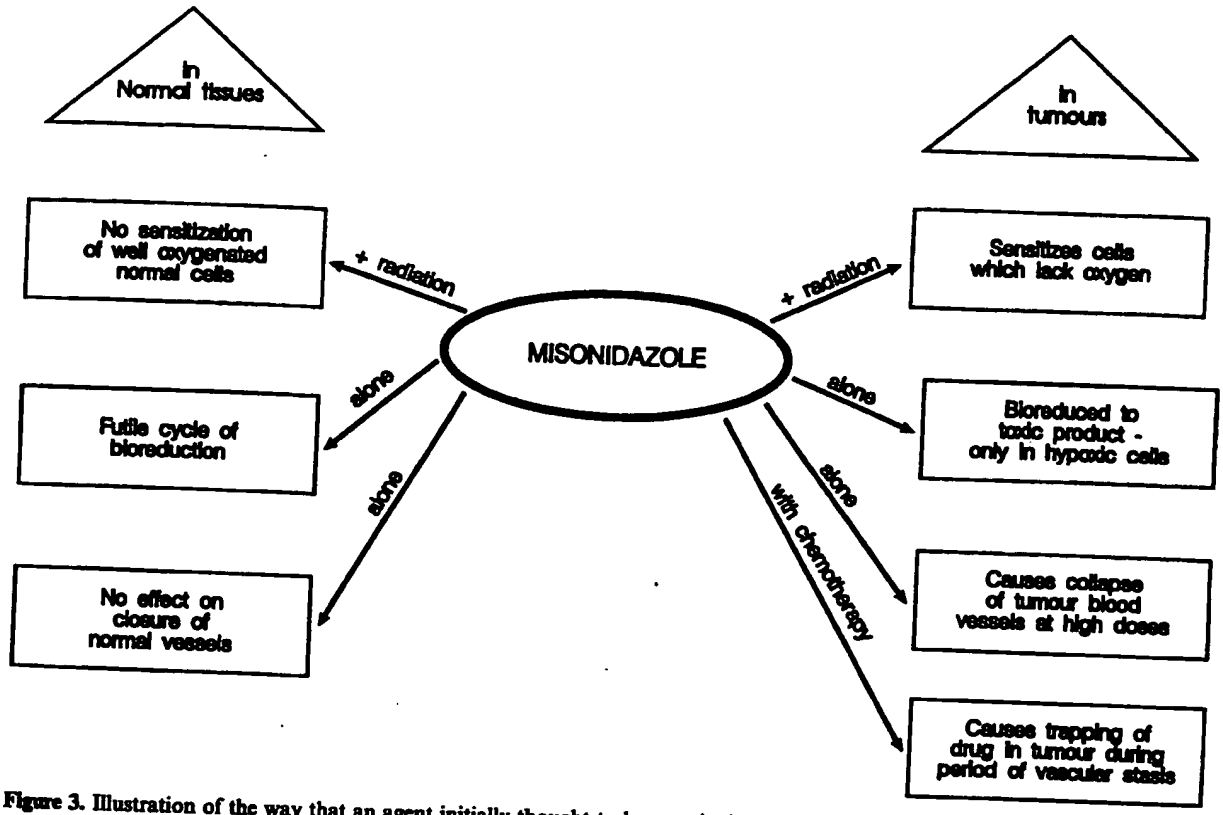

Figure 3. Illustration of the way that an agent initially thought to have a single well-understood mode of action has been shown subsequently to influence other aspects of tumour biology, which may also lead to a therapeutic advantage.

Prodrug activation

The converse of tumour sensitization is normal tissue radioprotection, which can be achieved with thiol compounds (which are proton donators) such as cysteine or cysteamine. WR2721 (Ethiophos) is a phosphorothiol, a less toxic version of cysteamine, which was originally developed in the United States as a whole body radioprotective agent for military purposes. It is a prodrug, with the thiol group blocked by a phosphate, which greatly reduces its systemic toxicity. For activation, and indeed for transport into or across cells, the compound must be dephosphorylated enzymatically. It was identified as a possible adjunct to radiotherapy because it was shown to give 10 times more radioprotection for bone marrow than for a rodent tumour (Yuhas & Storer, 1969). Radioprotection was observed subsequently in a range of normal tissues, particularly at short intervals after injection (Phillips et al, 1973). Studies using labelled WR2721 showed rapid and extensive uptake into normal tissues and slower and lower uptake in tumours, but the cause of this difference was ill understood. Yuhas (1980) suggested that the phosphatase activity might differ in tumour and normal cells but no such consistent pattern has emerged. However, a reduced alkaling phsophatase activity in tumour endothelium was identified when histochemical techniques were used in an attempt to stain tumour endothelium (Murray et al, 1989a) and this appears more likely to explain the delayed uptake of WR2721. The endothelium could effectively prevent passage of the prodrug across the vessel wall until a pool of the dephosphorylated compound, generated by alkaline phosphatases in normal vessels, would saturate the normal tissues and provide a systemic reservoir of the diffusible product which could then traverse the tumour endothelial barrier (Denekamp & Rojas, 1988). This concept of differential activation based on differences in surface enzymes in mature and immature endothelium or on differences in the quality of the blood in normal and tumour vessels may be a productive path to investigate for other prodrugs.

Hyperthermia

Studies with a novel form of cancer therapy, *i.e.* localized hyperthermia, in the late seventies and early eighties showed that the hypoxic tumour cells might be more sensitive to heat both because of their nutrient deprivation and because of the lower pH that results from anaerobic glycolysis. However, a much more important step was the realization that the newly formed vessels were themselves much more prone to heat damage than normal vessels.

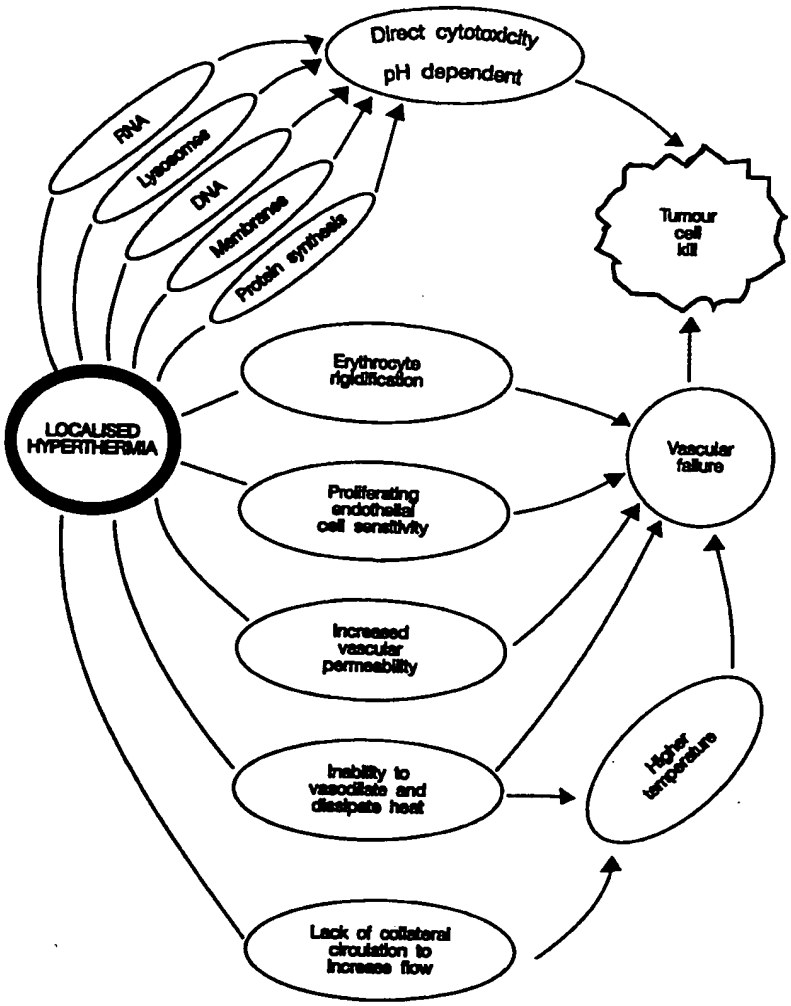

Figure 4. The effects of hyperthermia can be demonstrated as occurring directly on tumour cells and/or through the tumour vasculature. The key question is which mechanism will allow the maximum differential between tumour and normal tissue in man.

In most animal tumours, vessel occlusion and/or a reduction in blood flow are seen after a 30 min exposure to a moderate heat dose (41.5–43°C). A much higher temperature (46–47°C) is needed to produce the same effect in normal vessels (Eddy, 1980; Song, 1984, 1990; Reinhold et al, 1990). Thus a large therapeutic window has been identified which is not related to differences between normal and transformed cells, or even to the biochemical differences resulting from the poor vascular supply. The reason why vessel occlusion should occur at lower heat doses than in normal tissues is not clear, but rigidification of erythrocytes, adhesion of leucocytes to endothelium and exceptional thermal sensitivity of proliferating endothelial cells have all been identified as possible mechanisms (Endrich et al, 1979; Fajardo et al, 1985), (Figure 4).

Unfortunately this observation in animal systems has not led to widespread clinical benefit because the methods of administering deep heat are still a problem and pain limits the temperature that can be achieved in practice in patients. We have shown in rodents that slow growing tumours need a 0.5–1.0°C higher heat dose to achieve a thermal response than fast growing tumours (Hill et al, 1989a). The question therefore arises as to whether a temperature even higher than 43°C will be needed in slow-growing human tumours to lead to stasis. This would not be achievable without analgesia or local anaesthetics and would carry with it the risk of thermal burns to normal tissues. For superficial tissues a thermal burn might be an acceptable cost for massive tumour regression but thermal damage to bone or to neural tissue is clearly not.

The studies with heat summarized above were important because they illustrated that an agent may have been shown to cause direct tumour cell kill and there may be a rationale for expecting it to be more effective on tumour than normal cells, but nevertheless the therapeutic benefit may result from something totally

different, *i.e.* vascular occlusion leading to tumour cell starvation. Furthermore, it raised the question of whether the effectiveness of the agent might depend upon threshold dose characteristics for rodent tumours which do not match those of humans. It seems to be a characteristic property of vascular-mediated therapies that there is a threshold dose that must be exceeded in order to produce an effect. A corollary of this is that around the critical transition dose there is likely to be a distinction between responders and non-responders rather than the graded response which would occur if cell kill were random and progressive with dose.

A number of other forms of therapy, which were all developed as direct tumour cell killing agents or as immune-mediated effects targeted at malignant cells, have also been shown in recent years to cause vascular shutdown or to induce the characteristic pattern of focal necrosis. Some of these are discussed below.

Photodynamic therapy

Photodynamic therapy (PDT) depends upon the activation by visible light of a non-toxic compound, *e.g.* haematoporphyrin (photofrin), to release toxic activated oxygen species which kill the cell in which they have been formed. These extremely reactive free radicals are unlikely to live long enough to diffuse to adjacent cells and therefore they only damage the cell in which they are formed. It is evident under gross illumination that the compound, which is fluorescent, is retained preferentially in solid tumours some 3–4 days after administration. At first, this observation was attributed to leaky tumour blood vessels, *i.e.* altered permeability, but recently it has been suggested that the retention is actually within host cells, *e.g.* the endothelial cells of the newly formed tumour blood vesels or tumour-associated macrophages (Bugelski et al, 1981; Star et a, 1986; Korbelik et al, 1991). Reduced blood flow and vascular destruction have both been demonstrated and a discripancy between cell death *in vivo* and in excised tumours, consistent with ischaemia, has been measured. The undesirable side effects of damage to normal tissues have also been shown to be vascular-mediated. The reason for the apparent endothelial or macrophage localization is not understood but recent work has shown that at least part of the uptake is mediated by low density lipoprotein receptors (West et al, 1991). Proliferating endothelium has been shown to be more sensitive to PDT than plateau phase endothelial cells, or fibroblasts, or tumour cells. It is clear that differential host cell retention must be an important parameter when screening for alternative compounds to replace the existing haematoporphyrin derivatives. This cannot be done by simply screening uptake by tumour cells *in vitro*. At present the research emphasis in this field is almost entirely on the development of more active compounds, or those responding to more penetrating wavelengths of light. Hence, important new compounds may be missed and the therapeutic differential between tumour and normal retention observed with haematoporphyrins could be lost with the newer agents. Studies aimed at elucidating the mechanisms of uptake may lead to completely different drug design programmes (CML West, J V Moore & G Truscott, personal communication).

Chemotherapy

Most conventional cytotoxic drugs kill cells by causing damage to DNA, or by interfering with the normal processes of DNA replication or repair. Most pharmaceutical effort is aimed at developing drugs which are freely diffusible through tissue to reach the tumour cells most distant from the capillaries. The transvascular and interstitial movement of cytotoxic agents may be limited by the sinusoidal nature of many tumour blood vessels, the sluggish and intermittent blood flow and the high interstitial pressures which result from poor lymphatic drainage (Jain, 1988, 1989). If a drug is cycle specific a further problem is encountered because active proliferation is restricted to the first few cell layers around each vessel as a result of nutrient limitations (Tannock, 1970; Hirst & Denekamp, 1979). Thus the poor tumour vasculature creates several distinct problems in the effective use of cytotoxic drugs (Figure 5). Even though cures of solid tumours are uncommon if the tumours are treated only with chemotherapeutic regimes, significant tumour regression is seen in many instances. It seems appropriate to ask whether this could be the end result of endothelial cell kill leading to vascular-mediated damage instead of, or as well as, direct tumour cell kill. There is a high rate of cell division of endothelial cells in all solid tumours which could make them susceptible to proliferation specific cytotoxins. Endothelial cell death would expose basement membrane which could act as a focus for thrombus formation or for a site of leakage leading to oedema or haemorrhage. Chemical agents may also modify the activity of various host cells, increasing the production of vasoactive agents, *e.g.* nitric oxide, tipping the balance towards coagulation, *e.g.* by altering tissue factor production or by changing circulating blood cell adhesion to vessel walls. Any of these could lead to a decrease in tumour perfusion. If the chemical alters cardiac function, *e.g.* heart rate, stroke volume or blood pressure, there could also be regional redistribution of flow leading to a "steal" effect away from the tumour. A tremendous avalanche of tumour cell death could occur for each occluded capillary resulting from vascular occlusion at the site of endothelial cell depletion. Hence this could be an important target. It should be possible to investigate this by looking for hallmarks of vascular effects in tumours (Figure 6) or normal tissues (Figure 7) after chemotherapy.

Very few studies have been published of changes in vascular parameters after chemotherapy which reflects the lack of interest in this possibility. In the study of melphalan and misonidazole described above (Murray et al, 1987) where vascular parameters were measured a significant effect on blood flow was seen when melphalan was used alone, a bigger effect from miso alone and a massive and prolonged effect from the combination.

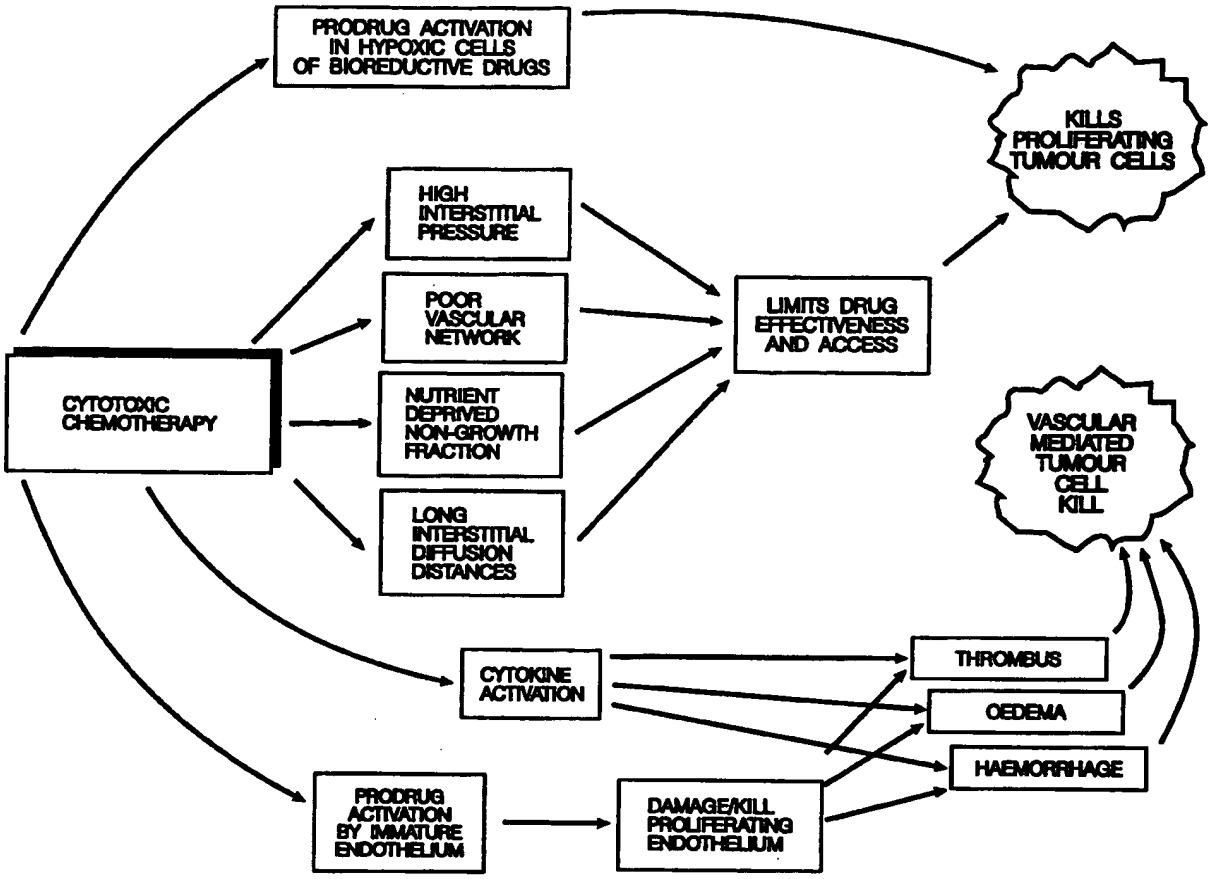

Figure 5. The effectiveness of conventional cytotoxic chemotherapy against tumour cells is limited by the poor tumour vasculature. However, some of the tumour responses observed may result from damage to or altered function of proliferating and immature endothelium.

Indirect evidence of a rapid host-mediated cytotoxity from conventional cytotoxic drugs which may be related to blood flow is available from a reanalysis of the studies of survival *in vitro* after excising tumours treated *in vivo* (Stephens & Peacock, 1978). For most of the drugs tested the yield of cells was high if tumours were harvested an hour or two after drug administration. However, this yield fell if the cells were harvested 24 h later when host effects could have led to rapid cell killing and removal. After the vinca alkaloids the cell yield fell to 25% of the original value and after other agents the values ranged from 50–75%. The timescale of this effect is compatible with rapid necrosis that is associated with vascular collapse. Studies are in progress to compare the growth delay with histological patterns and alterations in blood flow after a range of conventional cytotoxic drugs. Recently more direct evidence of antivascular activity of vinca alkaloids has come from a study of colon carcinomas in mice (Baguley et al, 1991). Haemorrhagic necrosis was observed within 8 h of vinblastine and colchicine. Sublines of the P388 tumour that were sensitive or resistant to the effects of these drugs when grown as perpitoneal ascites lost their differential sensitivity as solid subcutaneous tumours.

Two novel chemotherapeutic drugs have recently been taken into clinical trials based on the dramatic responses that had been seen in a range of rodent tumours. These are the Lipha drug Flavone Acetic Acid (LM975) and the Glaxo drug Phoquidone (GR3178). Both agents were shown to cause extensive haemorrhagic necrosis in solid mouse tumours, but no effect in leukaemias or lymphomas. Flavone acetic acid (FAA), developed originally as an anti-inflammatory agent, has no antimetabolite activity and only weak DNA binding properties. Its action has many of the hallmarks of vascular-mediated injury although these were not initially recognized as such.

In the initial screening process funded by NCI, FAA was found to be remarkably effective on all solid tumour models, but had no effect on lymphomas or leukaemias (Corbett et al, 1986). It was found to be completely ineffective when given immediately after

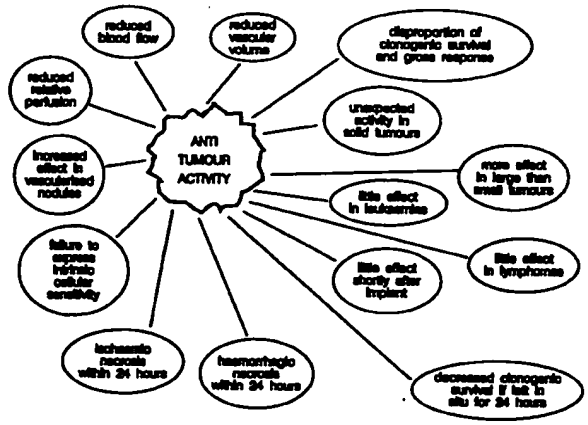

Figure 6. Features of tumour response to therapeutic agents that may indicate a vascular mechanism is involved.

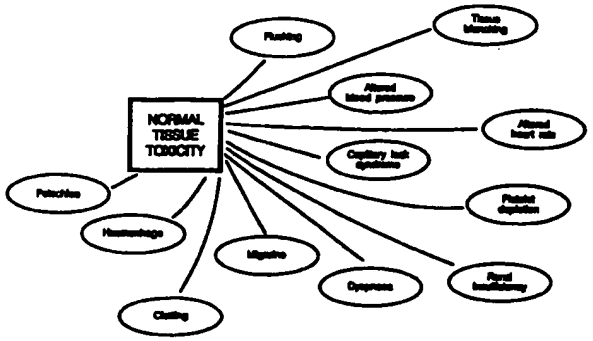

Figure 7. Side effects of anti-cancer agents that should be regarded as indicators that vascular changes are occurring, and which could be clues that anti-tumour activity may be indirect, mediated by vascular shut-down.

tumour cell inoculation, and became more effective when given to large established (*i.e.* vascularized) solid tumours (Bibby et al, 1987). It has little cytotoxic actions *in vitro*, which is incompatible with the effects *in vivo*, indicating that a host response must be involved. Tumour cell lines have been selected to be responsive or resistant to this agent in culture but these do not retain these sensitivity differentials *in vivo*.

A rapid haemorrhagic necrosis is visible within a few hours of administering FAA and this is accompanied by, or preceded by, a fall in tumour perfusion (Finlay et al, 1988; Hill et al, 1989). Decreased tumour perfusion has been detected within 15 min to 2 h, depending on the assay technique and this persists for at least 24 h. Hill et al (1991) have developed a panel of tumours showing a wide range of sensitivity to FAA. The FAA-sensitive tumours show extensive growth delay, or even long term local control, after single doses of the agent. The unresponsive tumours show little delay even after double the drug dose. The extent of reduction in blood flow is correlated with the growth delay. Gross necrosis can be scored within 24 h after a single dose of FAA and it is curious that all of the tumours show 85–95% necrosis (*i.e.* cell kill) even though the growth delay varies from a few days to almost 3 months. In a similar study of the Glaxo drug GR63178 a more variable level of necrosis is induced, ranging from 15–95% (Hill et al, 1991) in the same panel of tumours, but with this agent there is very little evidence of growth delay in any of the tumours (S A Hill, Gray Laboratory Annual Report 1991). Studies are under way to determine the cause of this variable response. At present, the strongest correlation seems to be that fewer macrophages are present in the sensitive than in the resistant tumours.

An interesting feature of FAA is that it is much more toxic (by a factor of 2–3), when it is administered to tumour-bearing mice, in a manner that is related to tumour load and also to the tumour's sensitivity to FAA. The increased toxicity can also be induced, though to a lesser extent, by other angiogenic stimuli, *e.g.* by surgery, or by the implantation of a surgical sponge. It therefore appears that a systemic effect is being induced both by the presence of a tumour, or by a simple network of immature blood vessels in granulation tissue. It seems likely that this reflects an altered pattern of coagulation. Changes in ocagulation parameters have been demonstrated within a short time after FAA injection. Initially the blood has a reducted clotting time, but after 24 h this is reversed, perhaps indicating depletion of the clotting precursors (Murray et al, 1989b). The coagulation cascade is modulated by tissue factors produced by endothelial cells, and these can be further altered by the presence of tumour cells, tipping the blood towards a procoagulant status (Murray, 1991; Murray et al, 1992). Evidence is also accumulation that this balance can be further tipped by the addition of tumour necrosis factor (TNF) to the system. The effect of FAA may even be mediated by immune effector cells, perhaps the local induction of TNF forming part of a chain reaction. This may explain the increased systemic toxicity in certain tumour-bearing mice. The production of EDRF (nitric oxide) and of serotonin are also implicated, since nitrates are detectable in the plasma and urine after FAA and the anti-tumour effects in mice can be blocked by competitors for nitric oxide synthase and by antagonists of serotonin (Zei et al, 1989; Thomsen et al, 1991; Baguley et al, 1991). A series of FAA analogues have anti-tumour effects that correlate with the level of nitrates in the plasma.

Recent studies have demonstrated very convincingly that FAA action is largely vascular-mediated but in addition there is an immunological component (Zwi et al, 1990). By implanting spheroids into the peritoneum they are able to study in the same animal the effect on similar sized tumours with and without a vascular network. The damage assessed histologically 18 h after FAA was much more marked in the vascularized tumours, with a differential effect even being detectable in the vascularized regions of the same spheroid. Even

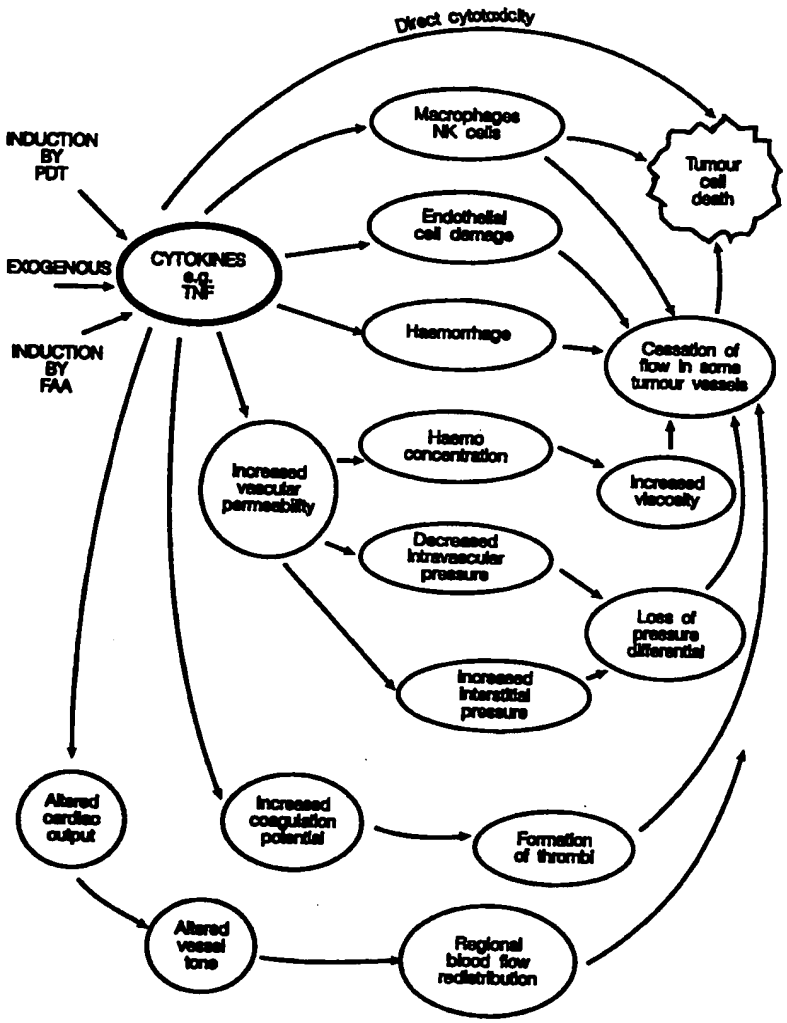

Figure 8. Illustration of the complexity of the responses that have been observed after cytokine therapy, whether administered as exogenous agents, *e.g.* TNF or IL2, or induced by chemical (FAA) or physicochemical (PDT) agents.

the non-vascularized spheroids showed much more effect than spheroids *in vitro* indicating the involvement of host cells. The immune status of the mouse has also been shown to be important by Bibby et al (1991). An FAA responsive tumour in syngeneic mice became unresponsive when implanted into nude mice, as are human tumours growing in immuno-suppressed or immuno-deficient mice. This is a further indication of the involvement of host cell activation in the anti-tumour effects of FAA (Figure 7).

FAA should perhaps be regarded as a lead compound for future therapies since it has indicated some potential new modes of vascular attack. However, it has been universally disappointing in the early clinical trials, in marked contrast to the dramatic effects seen in most rodent models. The early clinical studies showed that the main limiting side effect was severe hypotension, and also that decreased platelet aggregability was observed after 1–2 days. In order to minimize the troublesome hypotension, to maximize the chance of tumour cytotoxicity (based on *in vitro* data) and to avoid possible crystallization of FAA in the acid environment of kidney tubules, a protracted clinical schedule was developed. This seemed to differ in only minor ways relative to the acute bolus administration used in mice but it may be the key to the disappointing clinical results. The schedule consisted of slow infusion over 5–8 h in patients pre-treated with bicarbonate to avoid potential crystallization in the kidney tubules (Kerr et al, 1987, 1989; Kaye et al, 1990). If similar schedules are used in mice the dramatic tumour response seen with a bolus injection is either abolished or grossly reduced (Denekamp & Hill, 1991). This highlights the fact that, without an understanding of the mechanism of action, it is quite possible to reject drugs that are potentially effective in an alternative dose schedule. FAA in com-

bination with IL2 has recently been shown to have considerable anti-tumour activity in a subset of advanced melanoma patients (O'Reilly et al, 1991).

Cytokines

Cytokines are implicated as inducible mediators in a number of approaches to attacking tumours via immune activation. Cytokine induction may also be involved in some vascular damaging pathways especially after FAA and photodynamic therapy. Injection of the cytokines systemically, especially interferon, the interleukins and TNF has excited a lot of interest in them as cancer therapeutic agents. As with FAA and GR63178, their use in rodent tumours gave great promise which has not been matched by the clinical response with the schedules used in patients. Regional patchy necrosis was long ago identified in mice as the pattern of tumour response to endotoxin, TNF and interferon (*e.g.* Blocksma et al, 1982). A variety of studies have since shown endothelial reactivity, microvascular damage, changes in blood flow and/or altered responses to angiogenic stimuli with these and other cytokines (*e.g.* Bevilacqua et al, 1984, 1986; MacPherson & North, 1986; Sidky & Borden, 1987; Kotasek et al, 1988; North & Havell, 1988; Baguley et al, 1989; Dvorak & Gresser, 1989; Kalinowski et al, 1989).

These effects of cytokines seem more likely to involve alterations of endothelial cell function than endothelial cell kill. This may include changes in arachidonic acid metabolism, the control of coagulation by tissue factor and factor VIII production, platelet or neutrophil adhesion and production of nitric acid. It is clear that for a further understanding of these mechanisms a study of growth factors, coagulation control and of adhesion molecule expression of endothelial cells will be needed. Very few of these features can be studied in single cell types grown in culture but the complex biochemical cascades involving cells of different types, *e.g.* tumour cell, endothelial cell and macrophage, will need careful evaluation in appropriate coculture experiments or in the whole animal to allow their complete expression.

Summary

A body of evidence that vascular-mediated damage occurs in murine tumours after many existing forms of anti-tumour therapy is rapidly accumulating (see Gray Conference Proceedings edited by Moore & West, 1991). Rapid conventional screens of cells *in vitro* or using leukaemias of lymphomas will not detect this mode of action and such screens will therefore miss effective agents. A change in the approach to experimental cancer therapy is needed to ensure that this important new avenue is fully investigated. Solid tumours will need to be studied and the importance of specific tumour cell biochemistry (*e.g.* on tissue factor procoagulant activity), of endothelial status and the immunocompetence of the host are all likely to be important. It is a subject of considerable debate at present whether transplanted subcutaneous mouse tumours are adequate models and whether they will reflect the response of spontaneous tumours, or even of transplants into other sites. Xenografts are not likely to be appropriate if the immuno-suppressed hosts lack the cells needed for the cytokine component of the pathway.

The strategy of design and screening of new agents, for scheduling of existing agents and particularly the sequencing of adjunctive therapies are likely to be completely different for the "direct" tumour cell or "indirect" vascular-mediated approaches. It may eventually be appropriate to combine vascular manipulation with direct cytotoxicity aimed at malignant cells but the two mechanisms must be recognized as distinct entities and considered separately before attempting to coordinate them. It is important therefore to identify the "hallmarks" of vascular mediated injury and the means by which this can be distinguished from direct cell kill. These may be detectable in the tumour response but clues can also be gained from the side effects that are seen in normal tissues both with existing and with novel therapies (Figure 7).

The appeal of vascular-mediated ischaemic therapy is that it is systemic and will have the potential of being effective on any tumour with a newly evoked vascular network, *i.e.* of about 1 mm in diameter, but it will be even more effective on large tumours than on small. Thus it should affect both large primary tumours and disseminated small metastases. The studies with many different anti-cancer agents have illustrated the potential complexity of responses that can appear to cause tumour cell death by collapse or occlusion of the blood supply. They have also focused attention on features of disparate agents, *e.g.* TNF, FAA, PDT, which may share similar pathways.

No single feature of neovasculature can be highlighted as the sole route by which such antivascular therapy should be targeted. Rapid proliferation of the endothelial cells may prove to be a target, but it also influences differentiation characteristics, so that the immature cells will function abnormally. The permeability of these poorly formed vessels may lead to extravasation of proteins leading to increased interstitial pressures and by this means to an imbalance between intravascular and extravascular pressures and hence to collapse of the thin-walled vessels. Changes in systemic blood pressure, cardiac output, viscosity or coagulation and especially a redistribution of regional perfusion would all have differential effects in tumours and normal vessels. Clearly both vascular patho-physiology and the complexity of endothelial cell function and its imbalance in neovasculature will be important in understanding the mechanism of action of antivascular strategies. This very challenging boundary between oncology and a number of other medical and biological fields promises to lead to altered attitudes to existing therapies and the discovery of completely new classes of anti-cancer agents. The next decade should translate into clinical benefit for patients if the progress in this field continues to be as rapid as it has been in the late eighties. We must now determine what characteristics make one tumour more sensitive than another to agents

such as heat, PDT, cytokines and FAA, and learn how to extrapolate from those rodent tumours to the human.

Acknowledgment

This work has been entirely supported by the Cancer Research Campaign. Its evolution has only been possible because of stable funding of long-term projects over many years. It is intriguing that this field should have evolved from radiobiology since radiation is one of the agents that has only a minor component of vascular-mediated injury. I am grateful to my colleagues (especially Dr Sally Hill) for allowing me to present their data and to my secretary, Ann Hanford, for manuscript preparation and graphic design.

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